BIOCHIMICA
et
BIOPHYSICA ACTA

INTERNATIONAL JOURNAL OF BIOCHEMISTRY AND BIOPHYSICS
REVUE INTERNATIONALE DE BIOCHIMIE ET BIOPHYSIQUE
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VOL. 37 1960

ELSEVIER PUBLISHING COMPANY
AMSTERDAM

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ERRATUM

BIOCHIMICA ET BIOPHYSICA ACTA, VOL. **36** (1959)

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BIOCHIMICA ET BIOPHYSICA ACTA

ÉTUDE DE L'ACTION DU CHLORURE DE SODIUM SUR L'OXYHÉMOGLOBINE

II. INFLUENCE DE LA CONCENTRATION PROTÉIQUE SUR LA DISSOCIATION

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(Reçu le 20 Mars, 1959)

SUMMARY

Action of NaCl on oxyhemoglobin

II. Effect of protein concentration on the dissociation

Human oxyhemoglobin, even in diluted protein solutions, does not dissociate at pH 7 in a low ionic strength buffer.

If this buffer contains 1 *M* NaCl we observe a decrease of the molecular weight and of the sedimentation coefficient and an increase of the diffusion coefficient: the hemoglobin dissociates probably into half molecules, the dissociation depending on the protein concentration, in diluted solutions all the molecules being dissociated.

A simple equilibrium reaction between the whole hemoglobin molecule and the half molecules cannot entirely interpret the sodium chloride effect.

INTRODUCTION

La mesure par diffusion de la lumière de la masse moléculaire de l'oxyhémoglobine humaine a permis de mettre en évidence à pH 7 une action du chlorure de sodium[1]. Les résultats font penser qu'il se produit une rupture de la molécule en deux moitiés. Dans les conditions étudiées tout au moins (concentrations protéiques comprises entre 0.4 % et 0.1 % et concentration de NaCl supérieure ou égale à 0.5 *M*) un certain nombre de molécules restent entières ce qui pourrait s'interpréter soit par l'existence de deux sortes de molécules, soit par une réaction d'équilibre entre molécules entières et molécules dissociées.

Le but du présent travail est de compléter ces premiers résultats et de préciser l'action du sel par une étude systématique, en fonction de la concentration des solutions protéiques, des trois paramètres que nous avons choisis pour caractériser la molécule: masse moléculaire mesurée par diffusion de la lumière, constante de diffusion brownienne et constante de sédimentation.

Précisons que la préparation de l'oxyhémoglobine à partir de sang humain, le dosage et la conservation des solutions se font comme précédemment[1,2].

Les expériences ont été effectuées à pH 7 sur deux types de solutions d'oxyhémoglobine à différentes concentrations: (a) dans un tampon phosphates $M/45$; (b) dans un tampon phosphates $M/45$ additionné de NaCl 1 M.

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MESURES PAR DIFFUSION DE LA LUMIÈRE DE LA MASSE MOLÉCULAIRE

Rappelons que M (masse moléculaire moyenne en poids) est donnée par la formule:

$$\frac{1}{M} = \frac{2\pi^2 n^2}{N \lambda_0^4 R_B} I_B \left(\frac{dn}{dc}\right)^2 \left\{\frac{c}{I - I_0}\right\}_{c=0} = K \left(\frac{dn}{dc}\right)^2 I_B \left\{\frac{c}{I - I_0}\right\}_{c=0}$$

où n est l'indice du benzène à la longueur d'onde λ_0; λ_0 la longueur d'onde incidente dans le vide; N le nombre d'Avogadro; R_B le rapport de Rayleigh du benzène pour la longueur d'onde λ_0 ($16.3 \cdot 10^{-6}$ pour 5461 Å); I_B l'intensité diffusée par le benzène; I_0 l'intensité diffusée par le solvant; I l'intensité diffusée par la solution; dn/dc l'incément d'rindice de réfraction; c la concentration en g/cm^3.

$1/M$ est donc proportionnel à $c/(I - I_0)$.

Les mesures ont été effectuées dans des conditions identiques à celles de nos premières expériences[1]. Nous avons étendu le domaine initial de concentrations (0.1 g %–0.4 g %) vers les faibles concentrations (jusqu'à 0.01 g %) et vers les fortes concentrations (jusqu'à 1.2 g %)*.

L'expérience donne $c/(I - I_0)$ en fonction de c; M est obtenue par extrapolation pour $c \doteq 0$. A chaque concentration l'intensité diffusée est liée non seulement à M mais au deuxième coefficient du viriel B: $c/(I - I_0) \; \alpha \; 1/M + 2\,Bc$.

La Fig. 1 rend compte des résultats.

L'Oxyhémoglobine en solution dans un tampon phosphates M/45 *à pH 7*

Il n'y a pratiquement pas de variation de $c/(I - I_0)$ en fonction de la concentration protéique dans les solutions diluées d'oxyhémoglobine (la masse moléculaire est d'environ 68,000).

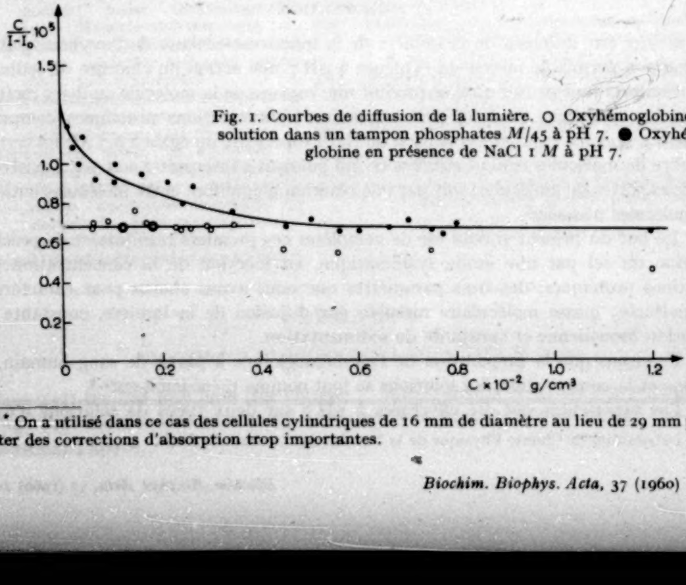

Fig. 1. Courbes de diffusion de la lumière. ○ Oxyhémoglobine en solution dans un tampon phosphates $M/45$ à pH 7. ● Oxyhémoglobine en présence de NaCl 1 M à pH 7.

---

* On a utilisé dans ce cas des cellules cylindriques de 16 mm de diamètre au lieu de 29 mm pour éviter des corrections d'absorption trop importantes.

L'Oxyhémoglobine en solution dans un tampon phosphates M/45 à pH 7 additionné de NaCl 1 M

Aux concentrations protéiques élevées ($c > 0.4\%$) le sel n'a pas d'action*. La valeur de $c/(I-I_0)$ est indépendante de la concentration et identique à la valeur extrapolée pour $c = 0$ dans le tampon sans NaCl. Dans le tampon seul, par contre, on observe dans ce domaine de concentrations une décroissance des valeurs de $c/(I-I_0)$ due aux interactions entre les molécules protéiques. Si l'on interprète cette variation par l'existence d'un deuxième coefficient du viriel, le fait que, dans NaCl 1 *M*, $c/(I-I_0) = f(c)$ soit une horizontale indique que les interactions moléculaires semblent alors négligeables. Dans ces conditions, puisque le deuxième coefficient du viriel est pratiquement nul dans NaCl 1 *M*, on peut calculer, pour chaque concentration protéique, la masse moléculaire qui est la moyenne en poids des espèces existant dans la solution. (Il est probable en effet que dans le cas de demi-molécules le deuxième coefficient du viriel demeure très faible.)

Pour des concentrations inférieures à 0.4% la variation de $c/(I-I_0)$ avec la concentration que l'on avait observée dans NaCl 1 *M* est donc due à la variation des quantités relatives d'oxyhémoglobine entière et dissociée. La dissociation provoquée par le sel serait une réaction d'équilibre entre molécules entières et demi-molécules. Le comportement de la protéine dans les solutions diluées ($c < 0.1\%$) semble le confirmer: $c/(I-I_0)$ augmente plus rapidement au fur et à mesure que c diminue. L'extrapolation pour $c = 0$ conduit à une masse moléculaire de 36,000 environ soit une valeur peu différente de la moitié de la masse moléculaire de l'oxyhémoglobine (68,000) ce qui correspondrait à une dissociation quasi totale.

Ce phénomène de dissociation mis en évidence par les mesures de diffusion de la lumière doit modifier d'autres paramètres moléculaires. Nous avons alors mesuré la constante de diffusion brownienne et la constante de sédimentation qui, dans le domaine où la dissociation se produit, c'est-à-dire aux faibles concentrations protéiques, peuvent être déterminées avec une précision suffisante.

Après avoir décrit pour chaque technique la réalisation expérimentale, nous donnons l'ensemble des résultats qui viennent confirmer ceux de diffusion de la lumière.

MESURES DE DIFFUSION BROWNIENNE

Dispositif expérimental

Nous avons utilisé pour mesurer la constante de diffusion D en solution diluée, une méthode décrite par DAUNE, FREUND ET SCHEIBLING[4] dont nous rappelons brièvement le principe et les caractéristiques essentielles.

On établit dans la cellule de diffusion une surface de séparation plane et horizontale entre le solvant et la solution (de concentration c_0). De part et d'autre de cette surface, à une distance a, passent les deux rayons interférents d'un interféromètre de Jamin. A la variation de la différence de chemin optique entre ces deux rayons, due au phénomène de diffusion, correspond le passage au cours du temps t de franges d'interférence ce qui se traduit par des variations d'éclairement de la photocathode d'un multiplicateur d'électrons. L'enregistrement continu du courant d'anode nous permet de suivre au cours du temps le phénomène de diffusion.

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* NICOLAÏEFF par la méthode de diffusion des rayons X aux petits angles[3] a également constaté que les courbes de diffusion déterminées sur des solutions protéiques de 2% à 10% sont identiques à pH 7 dans le tampon et dans le tampon additionné de NaCl 1 *M*.

Nous avons dû résoudre dans le cas de l'hémoglobine certaines difficultés techniques. Les solutions mêmes diluées sont colorées et absorbent fortement la lumière visible jusqu'à 6000 Å. On est donc obligé d'éclairer le dispositif interférentiel en lumière rouge; nous avons choisi la raie rouge principale du cadmium soit 6438 Å. En fait nous disposions d'une lampe à filament de tungstène de grande brillance et d'un monochromateur Zeiss réglé pour cette longueur d'onde. Si l'on veut un ordre d'interférence suffisamment élevé (de l'ordre de 100), les fentes d'entrée et de sortie doivent être fines; le flux lumineux est alors faible. D'autre part dans ce domaine spectral la sensibilité du photomultiplicateur IP 28 décroît fortement. Aussi avons-nous employé comme système d'enregistrement un galvanomètre de bonne sensibilité (10^{-9} A pour 1 mm à 1 m) associé à un suiveur de spot (SEFRAM). Les courants maxima mesurés étaient de l'ordre de $3 \cdot 10^{-7}$ A, mais, au prix d'une stabilisation très soignée de toutes les sources de tension, les fluctuations de courant ne dépassaient pas $3 \cdot 10^{-9}$ A.

Détermination de la constante de diffusion

L'intégration de l'équation de Fick conduit, dans le cas d'un corps pur, à l'équation suivante:

$$\log t^{3/2}/\delta t = \log aN/2\,(\pi D)^{\frac{1}{2}} - a^2/4\,Dt \qquad (1)$$

où δt représente l'intervalle de temps séparant le passage de deux franges consécutives*.

En portant la quantité $\log t^{3/2}/\delta t$ en fonction de $1/t$ on obtient une droite. On calcule D à partir de l'ordonnée à l'origine (D_1) ou de la pente (D_2). La constante D_2 reliée directement à des mesures de longueur et de temps peut être déterminée avec une précision supérieure à 0.5 %. Pour calculer D_1 il faut aussi connaître l'indice relatif de la solution par rapport au solvant dont la précision n'est jamais supérieure à 1 %.

Dans le cas d'un système polydispersé on obtient une courbe qui tend vers une droite quand $1/t$ devient suffisamment petit. Les valeurs D_1 et D_2 calculées à partir de cette droite limite ne sont plus égales; leur rapport permet de caractériser et dans certains cas de déterminer la polydispersité de l'échantillon[5]. Il est possible notamment de calculer les deux constantes de diffusion D_A et D_B dans un mélange binaire de deux constituants A et B[6]. Il faut remarquer que dans ce cas on a toujours

$$D_A > D_1 > D_2 > D_B$$

Les deux constantes D_1 et D_2 sont des valeurs extrapolées à concentration nulle, lorsque la variation de D avec c est faible et se fait suivant une loi linéaire.

Si la loi de variation de D avec c est quelconque, l'intégration de l'équation de Fick n'est en général plus possible et il est préférable de calculer D suivant une méthode due à BOLTZMANN[7]. On admet simplement que le phénomène de diffusion dépend uniquement de la variable $u = xt^{-\frac{1}{2}}$.

L'équation aux dérivées partielles

$$\frac{\partial c}{\partial t} = \frac{\partial}{\partial x}\left(D\,\frac{\partial c}{\partial x}\right)$$

---

* La différence de marche entre les deux rayons interférents correspond au temps 0 à N franges pour la longueur d'onde utilisée.

peut alors être remplacée par l'équation différentielle

$$-u\,\frac{dc}{du} = 2\,\frac{d}{du}\left(D\,\frac{dc}{du}\right) \tag{2}$$

En intégrant on obtient D sous forme d'une fonction de u.

$$D\,(u) = \frac{1}{2}\left(\frac{dc}{du}\right)^{-1}\int_u^{\infty} u\,\frac{dc}{du}\,du \tag{3}$$

Dans notre cas on exprime D en fonction de t et pour la valeur a de x ce qui conduit à la nouvelle expression

$$D\,(t) = \frac{a^2}{4t^{3/2}dc/dt}\int_0^t t^{-\frac{1}{2}}\,\frac{dc}{dt}\,dt \tag{4}$$

L'expérience nous donnant la valeur de dc/dt on peut donc par intégration graphique calculer $D\,(t)$ et par suite $D\,(c)$.

En réalité la variation de D est due à des influences simultanées de la concentration dans la région solvant ($c > c_0/2$) et dans la région solution ($c < c_0/2$), et la méthode n'offre d'intérêt que si cette influence est limitée à l'une ou l'autre de ces régions. En particulier à un équilibre entre deux ou plusieurs types de molécules se produisant au-dessous d'une certaine concentration, correspond une variation apparente de D avec c qu'il est alors facile de mettre en évidence.

Résultats expérimentaux

Oxyhémoglobine dans un tampon phosphates M/45 *à pH 7:* quelles que soient les concentrations initiales, les courbes de diffusion sont toutes des droites. On n'observe pas de variation de D avec la concentration et les rapports D_1/D_2 sont toujours très voisins de 1. On est donc, semble-t-il en présence d'une protéine homogène et qui ne subit aucune modification en solution très diluée.

En fait les valeurs des constantes de diffusion ne sont identiques que si elles correspondent à une même préparation. Si par contre on compare les résultats obtenus sur des préparations différentes, la dispersion des valeurs est beaucoup plus grande et bien supérieure aux erreurs d'expérience. C'est ainsi que sur 18 expériences de diffusion on trouve une constante de diffusion D_2 dont la valeur moyenne est de $6.86 \cdot 10^{-7}$ cm²/sec avec un écart moyen de ± 0.24 (valeur du reste en bon accord avec celle trouvée par différents auteurs[8,9]). Il est donc indispensable de ne comparer entre elles que des mesures physiques réalisées à partir d'une même préparation*.

Oxyhémoglobine dans un tampon phosphates M/45 *en présence de NaCl 1* M *à pH 7:* chaque expérience de diffusion doit être précédée d'une longue dialyse car le moindre déséquilibre dans les concentrations salines perturbe le phénomène**.

Les mesures de diffusion de la lumière semblent indiquer qu'il n'y a pas de modification de la molécule pour des concentrations protéiques supérieures à 0.4 %.

Pour vérifier cette hypothèse, nous avons mis en contact deux solutions protéiques à différentes concentrations. On a ainsi étudié la diffusion d'une solution à 1.2 % contre une solution à 0.6 %, et celle d'une solution à 0.9 % contre une solution à 0.3 %. Les points expérimentaux s'alignent sur une même droite (Fig. 2) et la constante D_2 est égale à $6.82 \cdot 10^{-7}$ cm²/sec. On peut en conclure que dans le domaine de concentration compris entre 1.2 % et 0.3 % la diffusion brownienne ne permet de déceler aucune modification de la molécule d'hémoglobine. La constante D_2 mesurée correspond bien pour cet échantillon à celle de l'oxyhémoglobine normale.

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* Rappelons que D est exprimé à 20° C par rapport à l'eau pure.

** Nous avons vérifié que la dialyse ne change rien aux résultats des mesures de diffusion de la lumière.

Si maintenant on étudie la diffusion d'une solution à 1.2 % contre le dialysat, les points expérimentaux se placent sur une courbe (Fig. 2), ce qui indique la présence d'un constituant plus rapide au début de la diffusion (c'est-à-dire dans le domaine des faibles concentrations). La pente de la droite limite conduit pour D_2 à une valeur de $6.77 \cdot 10^{-7}$ cm²/sec. Le fait de trouver une valeur très voisine de la constante de diffusion de l'hémoglobine normale nous permet d'affirmer que la courbure observée n'est pas due à un effet de polydispersité et que par conséquent la solution ne se présente pas comme un mélange binaire de proportion fixe. Comme d'autre part la région solution ($c > 0.4$ %) n'est le siège d'aucune dissociation moléculaire, nous pouvons appliquer à la région solvant la méthode de BOLTZMANN et chercher à déterminer directement la variation de D avec la concentration.

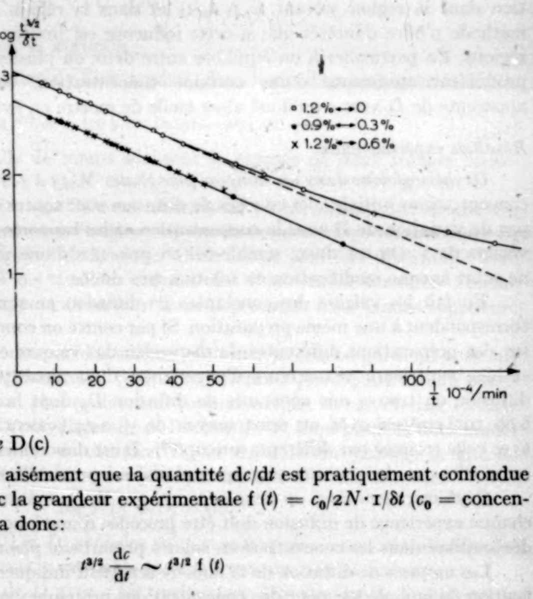

Fig. 2. Courbes de diffusion brownienne de l'oxyhémoglobine en tampon phosphates $M/45$ + NaCl 1 M, pH 7. La droite correspond à deux diffusions de solution contre solution (1.2 % contre 0.6 %; 0.9 % contre 0.3 %). La courbe correspond à la diffusion d'une solution à 1.2 % contre le dialysat.

Etablissement de la courbe D(c)

Méthode: on montre aisément que la quantité dc/dt est pratiquement confondue (au 2ème ordre près) avec la grandeur expérimentale $f(t) = c_0/2N \cdot 1/\delta t$ (c_0 = concentration au temps 0). On a donc:

$$t^{3/2} \frac{dc}{dt} \sim t^{3/2} f(t)$$

$$t^{-\frac{1}{2}} \frac{dc}{dt} \sim t^{-\frac{1}{2}} f(t)$$

On peut donc d'après (4) calculer $D(t)$ en intégrant graphiquement entre 0 et t la fonction $t^{-\frac{1}{2}} f(t)$ et en multipliant le résultat par $a^2/4\, t^{3/2} f(t)$.

On a de même $c(t)$ par intégration graphique de $f(t)$.

En fait le premier temps t_0 mesurable correspond au passage de la première frange et les courbes $f(t)$ et $t^{-\frac{1}{2}} f(t)$ doivent être extrapolées entre t_0 et 0. Les valeurs de D correspondant aux concentrations comprises entre 0 et $c(t_0)$ sont donc très douteuses et nous limiterons la courbe expérimentale à l'abscisse $c\ (t_0)$. De plus l'erreur relative sur $D(c)$ diminue au fur et à mesure qu'on s'éloigne de $c\ (t_0)$.

Résultats: dans le cas de l'hémoglobine et dans les conditions d'observation [$\lambda = 6438$ Å, $\mathrm{d}n/\mathrm{d}c = 0.198$, e (épaisseur de la cellule de diffusion) = 2.5 cm]

$$c\ (t_0) = \frac{c_0}{2N} = \frac{\lambda}{2e\ \mathrm{d}n/\mathrm{d}c} = 6.5 \cdot 10^{-5}\ \mathrm{g/cm^3}.$$

On ne peut donc espérer obtenir les valeurs de $D(c)$ pour des concentrations inférieures à 0.0065 %.

Nous avons d'abord constaté que dans le cas de la diffusion dans le tampon seul d'une solution de 1.2 % contre le dialysat l'application de la méthode de Boltzmann nous donnait pour $D(c)$ une droite pratiquement horizontale de 0.0065 % à environ 0.15 %.

Dans le cas de la diffusion en présence de sel (NaCl 1 *M*) on obtient pour $D(c)$ une courbe (Fig. 3) qu'il est difficile d'extrapoler à concentration nulle. Cependant pour la concentration $c\ (t_0) = 0.0065$ % on trouve une constante de diffusion de $9.46 \cdot 10^{-7}$ cm²/sec, alors que celle de l'hémoglobine normale est d'environ $6.80 \cdot 10^{-7}$ cm²/sec pour la préparation étudiée.

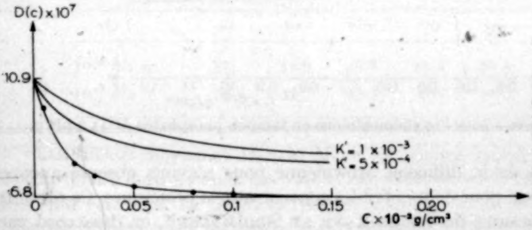

Fig. 3. Variation de *D* avec la concentration. Les points expérimentaux sont obtenus par la méthode de BOLTZMANN. Les deux courbes théoriques correspondent à deux valeurs de la constante d'équilibre *K'*.

On est donc bien, dans ce domaine de concentration protéique, en présence de molécules plus petites que celles d'hémoglobine normale. Nous verrons comment on peut interpréter au moins qualitativement l'allure de la courbe obtenue au moyen d'hypothèses simples.

MESURES DE VITESSE DE SÉDIMENTATION

Dispositif expérimental et mesure de s

Nous avons utilisé une ultracentrifugeuse Spinco, modèle E, équipée du système R.T.I.C. de contrôle et de régulation de la température. Toutes les sédimentations ont été effectuées à 59,780 tours/min et à 20° C ± 0.1. Nous avons employé concurremment deux méthodes optiques d'observation: (a) celle classique de Philpot-Svensson pour des concentrations supérieures à 0.2 % (b) une méthode d'absorption en lumière bleue monochromatique ($\lambda = 4358$ Å) qui nous a permis d'étudier des concentrations très faibles (jusqu'à 0.006 %).

L'image de la cellule d'ultracentrifugeuse se forme sur une plaque photographique (Kodak-Super-Speed Ortho). Les conditions d'exposition correspondent à la partie linéaire de la courbe de l'émulsion. Le cliché obtenu est développé avec un révélateur tamponné (Kodak D 76 d) et ensuite étudié sur un microphotomètre Joyce à double faisceau. On obtient directement une courbe de densité optique, c'est-à-dire (à une constante près) la répartition de la concentration en fonction de l'abscisse dans la cellule. La constante de sédimentation s est mesurée pour le point dont l'ordonnée est égale à la moitié de la concentration initiale.

Résultats expérimentaux

Oxyhémoglobine dans un tampon phosphates M/45 *à pH* 7: la Fig. 4 représente en fonction de c les différentes valeurs de s obtenues par l'une ou par l'autre méthode optique. Les résultats expérimentaux sont plus dispersés dans le cas de la méthode d'absorption. De plus il n'a pas été possible de réaliser toutes les sédimentations sur le même échantillon. Cependant la valeur moyenne obtenue pour des concentrations comprises entre 0.23 % et 0.6 %, soit 4.27 S, est nettement plus faible que celle mesurée pour 0.0064 % $< c <$ 0.2 %, soit 4.53 S. Il y a donc une légère variation de s avec la concentration. Nous pouvons admettre pour $s^{0}_{\text{eau, 20}}$ la valeur de 4.6 S ± 0.1.

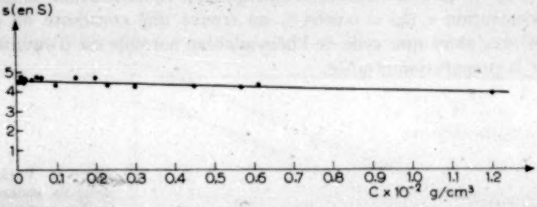

Fig. 4. Variation de s avec c pour l'oxyhémoglobine en tampon phosphates $M/45$ à pH 7.

Comme dans le cas de la diffusion brownienne nous n'avons observé aucune modification de la molécule pour de très faibles concentrations protéiques. Ce résultat est en accord avec les mesures de SCHACHMANN ET SCHUMAKER[10], en désaccord par contre avec celles de HUTCHINSON ET VINOGRAD[11].

Si dans la formule de Svedberg

$$M = \frac{R\,T\,s}{D\,(1 - V\rho)}$$

nous adoptons les valeurs suivantes:

$$s = 4.6 \cdot 10^{-13}$$
$$D = 6.86 \cdot 10^{-7}\ \text{cm}^2/\text{sec}$$
$$V = 0.749$$

On trouve $M \sim 65{,}000$ ce qui est en excellent accord avec la valeur moyenne de 68,000, déterminée par diffusion de la lumière, compte tenu des erreurs de mesure.

Oxyhémoglobine dans un tampon phosphates M/45 *additionné de NaCl 1* M *à pH 7*: la Fig. 5 représente les variations de s en fonction de la concentration en protéine. Les points relatifs aux concentrations 0.01 %–0.02 % et 0.06 % correspondent à des mesures par absorption.

Au lieu d'avoir une droite légèrement inclinée comme dans le cas de l'hémoglobine normale les valeurs de s diminuent à partir de 0.4 % et se placent sur une courbe.

Bien que l'extrapolation soit incertaine on peut admettre pour $s^{0}_{\text{eau, 20}}$ une valeur comprise entre 2.5 S et 2.6 S. Cette valeur est à rapprocher de celle obtenue par FIELD ET O'BRIEN dans le cas de la dissociation de l'hémoglobine à pH acide[8].

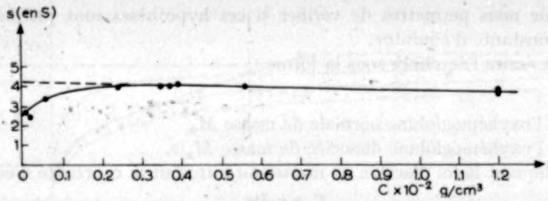

Fig. 5. Variation de s avec c pour l'oxyhémoglobine, en présence de NaCl 1 M à pH 7.

Comme l'ont signalé **FIELD ET OGSTON**[12] on ne peut déceler aucune dissymétrie de la courbe de gradient d'indice. Cette apparente homogénéité correspond à un équilibre rapide entre les deux constituants. La constante de sédimentation mesurée est évidemment une valeur moyenne. On peut cependant essayer de combiner les valeurs de s et D et calculer une masse moléculaire moyenne pour chaque concentration en supposant que le volume spécifique partiel reste inchangé.

Dans le tableau ci-dessous on a comparé, en fonction de c, les masses moléculaires calculées à partir de s et D, et celles mesurées par diffusion de la lumière.

c%	0.2	0.1	0.05	0.03	0.02	0.01	0.005
$10^{-3} M_{s,D}$	57	55.6	46.8	41.5	36.1	26.9	26
$10^{-3} M_w$	53.5	49	45.5	42.7	41	39	37

Les valeurs moyennes $M_{s,D}$ et M_w n'ont aucune raison d'être les mêmes, mais on doit tendre dans les deux cas vers la même masse moléculaire quand la concentration tend vers zéro. La polydispersité du milieu ne peut donc expliquer l'écart considérable observé pour les faibles concentrations. La différence est due uniquement aux valeurs trop élevées de D et nous verrons dans la discussion des résultats comment interpréter ce phénomène.

DISCUSSION DES RÉSULTATS

De l'ensemble des expériences il apparaît que la molécule d'oxyhémoglobine humaine en solution diluée à pH 7 reste entière dans un milieu de faible force ionique.

Par contre, en présence de NaCl 1 M, il se produit une scission de la molécule qui se traduit par une diminution de la masse moléculaire et de la constante de sédimentation et une augmentation de la constante de diffusion brownienne. Le phénomène dépend de la concentration protéique, ce qui conduit en première approximation à envisager cette dissociation comme une réaction d'équilibre entre molécules entières A et molécules dissociées B que nous supposons de masse moitié (*cf.* à ce sujet **FIELD, O'BRIEN ET OGSTON**[8, 12] ainsi que **HASSERODT ET VINOGRAD**[13]).

Calcul de la masse moyenne M_w en fonction de la concentration

Comme la diffusion de la lumière permet d'établir une courbe de variation de M avec c, il est intéressant de calculer cette courbe à partir d'hypothèse très simples faites sur l'équilibre de dissociation. La comparaison des courbes théoriques et expérimentale nous permettra de vérifier si ces hypothèses sont justifiées et d'en déduire la constante d'équilibre.

On peut écrire l'équilibre sous la forme

$$A \rightleftharpoons 2B \tag{5}$$

A désignant l'oxyhémoglobine normale de masse M_0
B désignant l'oxyhémoglobine dissociée de masse $M_0/2$.

En appliquant la loi d'action de masses on introduit la constante d'équilibre K:

$$K = n_B^2/n_A \tag{6}$$

n_A et n_B étant respectivement le nombre de moles de A et de B.

Nous ferons intervenir les concentrations en poids c_A et c_B définies par

$$\begin{aligned} c_A &= M_0\, n_A \\ c_B &= \frac{M_0}{2}\, n_B \end{aligned} \tag{7}$$

et la nouvelle constante d'équilibre

$$K' = c_B^2/c_A$$

qui est reliée à K par

$$K' = K \cdot M_0/4 \tag{8}$$

La masse moléculaire moyenne en poids M_w est définie dans ce cas par

$$M_w = \frac{M_0}{2}\left(\frac{c_B + 2c_A}{c}\right)$$

ou

$$M_w = \frac{M_0}{2}\,(1 + c_A/c) \tag{9}$$

(c = concentration totale d'oxyhémoglobine = $c_A + c_B$)

On a d'autre part

$$c_A = c + \frac{K'}{2} - \frac{K'}{2}\left(1 + \frac{4c}{K'}\right)^{\frac{1}{2}} \tag{10}$$

d'où finalement:

$$M_w = M_0\left\{1 + \frac{K'}{4c}\left[1 - \left(1 + \frac{4c}{K'}\right)^{\frac{1}{2}}\right]\right\} \tag{11}$$

Lorsque $c \to 0$ l'expression se simplifie

$$M_w \sim \frac{M_0}{2}\left(1 + \frac{c}{K'}\right) \tag{12}$$

La masse moléculaire tend vers $M_0/2$ et la variation est linéaire pour les faibles concentrations.

Pour des valeurs arbitraires de K' on peut donc construire une série de courbes $M = \mathrm{f}(c)$ et voir si l'une des courbes ainsi calculées se superpose à la courbe $M = \mathrm{f}(c)$ déduite de l'expérience.

Sur la Fig. 6 on voit que pour $K' = 5 \cdot 10^{-4}$ g/cm^3 ($K \sim 3 \cdot 10^{-8}$) les deux courbes sont superposées pour $0 < c < 0.2\,\%$. Pour les concentrations plus élevées l'accord n'est pas bon et la courbe calculée est au dessous de la courbe expérimentale, ce qui semble indiquer un mécanisme plus complexe que le schéma proposé.

Calcul de la constante de diffusion D *en fonction de* c

Après avoir interprété la variation apparente de M_w observée par diffusion de la lumière par un équilibre entre deux formes d'hémoglobine, il était intéressant d'introduire le même schéma dans les mesures de diffusion brownienne, pour tenter d'expli-

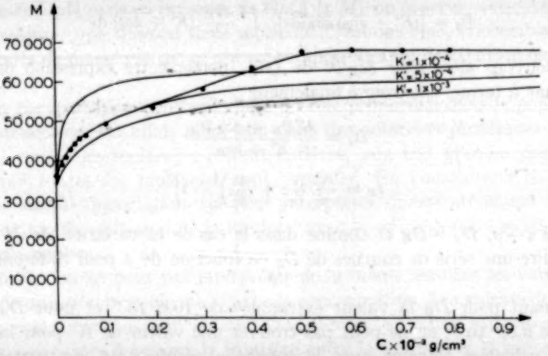

Fig. 6. Oxyhémoglobine en présence de NaCl $1 M$ à pH 7: Comparaison entre la courbe expérimentale de variation de M avec c, obtenue par diffusion de la lumière ● et les courbes calculées pour diverses valeurs de K'.

quer la courbe de variation de D avec c, obtenue avec la méthode de BOLTZMANN.

Nous supposons comme précédemment que l'équilibre entre la forme A (hémoglobine normale) et B (hémoglobine dissociée) s'écrit

$$A \rightleftharpoons 2\ B$$

avec

$$c_B^2 = K'\, c_A \tag{13}$$

c_A et c_B étant les concentrations en poids.

Nous supposons de plus que la vitesse de dissociation est grande par rapport à la vitesse de diffusion ce qui semble justifié par les résultats de sédimentation. Dans ces conditions l'équilibre est réalisé à chaque instant et on a la superposition de deux flux de diffusion satisfaisant chacun à la deuxième équation de Fick puisque la constante de diffusion est pratiquement indépendante de c dans le domaine de concentration étudié.

On a ainsi

$$\frac{\partial c}{\partial t} = D_A\, \partial^2 c_A/\partial x^2 + D_B\, \partial^2 c_B/\partial x^2 \tag{14}$$

avec

$$c = c_A + c_B \tag{15}$$

A partir de (13) et (14) on peut écrire l'équation de diffusion relative au constituant B

$$(K' + 2c_B)\, \frac{\partial c_B}{\partial t} = (K'D_B + 2c_B\, D_A)\, \partial^2 c_B/\partial x^2 + 2D_A\, (\partial c_B/\partial x)^2 \tag{16}$$

Dans la méthode de BOLTZMANN on définit D à partir de la première équation de Fick par la relation:

$$D_x = (\partial c/\partial x)^{-1} \int_{\infty}^{x} \partial c/\partial t \cdot \mathrm{d}x \tag{17}$$

Comme d'après (13) et (14) on a:

$$c = \frac{c_B^2}{K'} + c_B \tag{18}$$

on peut remplacer c dans l'expression de D_x par (18). On obtient ainsi la valeur du coefficient mesuré expérimentalement en fonction de la concentration c_B de l'espèce B

$$D_x = \left\{(K' + 2c_B)\,\partial c_B/\partial x\right\}^{-1} \int_{\infty}^{x} \frac{\partial c_B}{\partial t}\,(K' + 2c_B)\,\mathrm{d}x \tag{19}$$

Nous pouvons alors tirer $\delta c_B/\partial t$ de (16), porter cette expression dans (19) et intégrer terme à terme. On trouve finalement

$$D_x = \frac{K'D_B + 2c_B D_A}{K' + 2c_B} \tag{20}$$

avec

$$c_B = \frac{K'}{2}\left\{\left(1 + \frac{4c}{K'}\right)^{\frac{1}{2}} - 1\right\}$$

Lorsque $c \rightarrow 0$, $D_x \rightarrow D_B$ et comme dans le cas de la variation de M avec c, on peut construire une série de courbes de D_x en fonction de c pour différentes valeurs de K'.

En prenant pour D_B la valeur extrapolée de $10.9 \cdot 10^{-7}$ et pour D_A la valeur habituelle de $6.80 \cdot 10^{-7}$, on ne peut pas trouver une valeur de K' pour laquelle une des courbes calculées coïncide avec la courbe expérimentale. En particulier pour $K' = 5 \cdot 10^{-4}$ g/cm^3 il y a un désaccord complet (Fig. 3).

Il semble donc que les valeurs de $D(c)$ pour les concentrations très faibles soient systématiquement trop grandes. Nous avions déjà fait cette remarque lors du calcul de la masse moléculaire à partir de s et D.

Il est cependant possible d'utiliser l'expression (20) en supposant connue la constante K' (soit $5 \cdot 10^{-4}$ g/cm^3) et en déterminant D_B à partir des premières valeurs de $D(c)$ ($0.03\,\% < c < 0.1\,\%$).

On trouve alors pour D_B une valeur moyenne de $7.9 \cdot 10^{-7}$. La courbe $D(c)$ calculée s'écarte bien entendu de la courbe expérimentale pour les très faibles valeurs de c.

Si l'on attribue au constituant B les valeurs $D_B = 7.9 \cdot 10^{-7}$ et $s_B = 2.6 \cdot 10^{-13}$ on trouve $M_B = 32{,}000$ ce qui correspond bien à une molécule d'hémoglobine de masse moitié.

Les valeurs très élevées de la constante de diffusion pour les concentrations très faibles semblent dues à un phénomène de migration des ions à travers la surface initiale de séparation solvant-solution. En effet, bien que l'équilibre ionique soit atteint après une dialyse prolongée, la dissociation de la molécule doit modifier la répartition des ions au voisinage immédiat de la surface de séparation. Il en résulte un flux de diffusion supplémentaire dû aux petits ions et par suite un phénomène d'entraînement des molécules d'hémoglobine (dissociées ou non). Comme la vitesse de diffusion des ions est beaucoup plus grande que celle de la protéine, la constante moyenne mesurée est systématiquement trop grande tant que l'équilibre ionique n'est pas rétabli.

CONCLUSIONS

Nous pouvons résumer comme suit les résultats essentiels tirés de l'étude du comportement de l'oxyhémoglobine humaine en solution à pH 7 dans des milieux de faible force ionique ($\mu \sim 0.05$) et de force ionique élevée ($\mu \sim 1$):

1. A pH 7 en tampon dilué (phosphates $M/45$) la molécule d'hémoglobine reste entière même pour des concentrations protéiques très faibles ($\sim 0.005\,\%$). La masse moléculaire moyenne déduite des mesures de diffusion de la lumière $M_w = 68{,}000$ est identique, aux erreurs près, à la masse calculée à partir des constantes de diffusion et de sédimentation soit $M_{s,D} = 65{,}000$.

2. Au même pH mais en présence de NaCl (1 *M*) on observe, avec les trois techniques de mesure, une scission de la molécule d'hémoglobine, vraisemblablement en deux molécules de masse moitié, ce qui est compatible avec la structure de la molécule entière[14, 15].

Mais la concentration protéique joue un rôle primordial dans le pourcentage de molécules dissociées. En effet, alors que dans des solutions protéiques concentrées ($c > 0.4\,\%$) toutes les molécules restent entières, aux très grandes dilutions il est probable que toutes les molécules sont rompues. En considérant ce phénomène comme une simple dissociation, on peut interpréter quantitativement la variation de M_w (mesurée par diffusion de la lumière) avec la concentration et en déduire une constante d'équilibre.

Par contre, on ne peut pas interpréter de la même manière les variations de la constante de diffusion brownienne *D* avec la concentration. Les valeurs de *D* mesurées aux faibles concentrations sont en effet trop élevées, en raison sans doute d'une migration des petits ions à travers la frontière entre la solution et le solvant, car cette migration entraîne les molécules protéiques.

L'action du sel ne s'interprète donc pas uniquement par une réaction d'équilibre. Le schéma proposé pour la dissociation de l'hémoglobine n'est qu'une première approximation et n'explique plus les résultats expérimentaux de la diffusion de la lumière au-dessus de 0.2 %.

Dans une même hémoglobine il pourrait y avoir des molécules de stabilité différente. Des auteurs ont du reste signalé que l'hémoglobine serait constituée de plusieurs espèces moléculaires[16–18] qui ne réagiraient pas de façon identique à l'action d'un même agent[19].

La rupture par le sel ne peut atteindre que des liaisons relativement fragiles de la molécule. La dilution en tampon seul ne provoque pas la rupture de l'hémoglobine mais pourrait affaiblir certaines liaisons de la molécule. En solution diluée, toutes les molécules seraient sensibles à l'action du sel.

La nature de la liaison entre les deux moitiés de la molécule ne pourra être précisée que par une étude en fonction de la force ionique du milieu ainsi qu'en fonction de la température.

RÉSUMÉ

A pH 7 en solution dans un tampon de faible force ionique la molécule d'oxyhémoglobine humaine reste entière même aux faibles concentrations protéiques.

Si ce tampon est additionné de NaCl 1 *M*, la molécule se scinde, vraisemblablement en deux moitiés. Cette dissociation, qui se traduit par une diminution de la masse moléculaire et de la constante de sédimentation et par une augmentation de la constante de diffusion brownienne, dépend de la concentration protéique. Elle n'a pas lieu en solution concentrée; elle devient à peu près totale en solution diluée.

Une simple réaction d'équilibre entre molécules entières et molécules de masse moitié ne rend pas entièrement compte de l'action du chlorure de sodium.

REMERCIEMENTS

L'un de nous (N. BENHAMOU) tient à exprimer ici toute sa gratitude à Monsieur le Professeur SADRON qui, à plusieurs reprises, a bien voulu lui offrir l'hospitalité de son laboratoire et qui a permis la réalisation de ce travail.

Nous désirons remercier tout particulièrement Monsieur le Professeur **Benoît** qui au cours de cette étude nous a donné de précieux conseils, et Madame G. **Pouyet** pour son amical concours lors des mesures de sédimentation.

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ACTION SPECTRA FOR FLUORESCENCE EXCITATION OF PYRIDINE NUCLEOTIDE IN PHOTOSYNTHETIC BACTERIA AND ALGAE

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(Received March 23rd, 1959)

SUMMARY

An apparatus is described for the determination of u.v. action spectra of fluorescence excitation. Excitation spectra for the blue fluorescence increase caused by photosynthetic illumination of photosynthetic bacteria and algae have been found to be very similar to the excitation spectrum of DPNH bound to yeast alcohol dehydrogenase. The results support previous proposals based on fluorescence spectra that illumination causes the reduction of intracellular pyridine nucleotide bound to protein constituents.

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INTRODUCTION

In previous studies[1,2] of changes in blue fluorescence caused by photosynthetic irradiation of photosynthetic bacteria and algae, the fluorescence changes have been attributed to intracellular changes in bound pyridine nucleotide on the basis of the spectra of these changes and their similarity to the fluorescence spectra of DPNH (reduced diphosphopyridine nucleotide) bound to various dehydrogenases. In the present investigation this interpretation has been tested by determining action spectra of fluorescence excitation. Excitation spectra have been obtained for the light-induced fluorescence increase in two species of photosynthetic bacteria and two species of algae. For purposes of comparison the fluorescence excitation spectra for the DPNH-yeast ADH (alcohol dehydrogenase) complex and free DPNH have also been determined.

APPARATUS

The set-up for the determination of action spectra (260–400 mμ) of fluorescence excitation is shown schematically in Fig. 1. Lens L_1 places a slightly defocused image of the Xenon arc at the entrance slit of the monochromator. The exit slit of the monochromator is imaged on the sample cuvette by 1/2 L_1. A quartz plate (thickness 1 mm) in the excitation beam directs about 8 % of the incident light to a phototube which monitors the intensity. The fluorescing portion of the sample is imaged on the photocathode of the photomultiplier by L_3. The tungsten lamp in the light condenser system illuminates the rectangular stop uniformly, and lens L_4 images the stop on the sample cuvette.

The 1000 watt Xenon lamp (Osram XBO 1001) is operated on batteries. The quartz lenses L_1 and L_2 are the standard entrance slit optics of the Bausch & Lomb

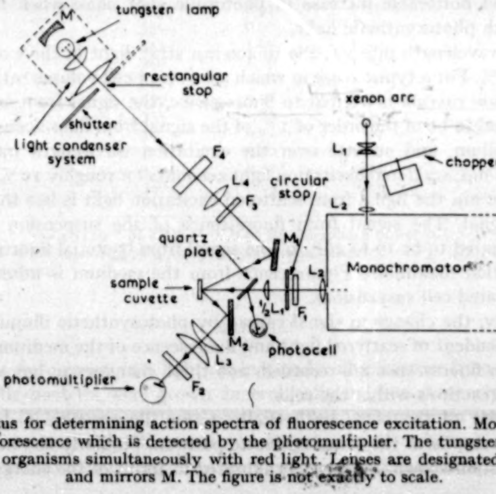

Fig. 1. Apparatus for determining action spectra of fluorescence excitation. Monochromatic u.v. light excites fluorescence which is detected by the photomultiplier. The tungsten lamp irradiates photosynthetic organisms simultaneously with red light. Lenses are designated L, filter sets F, and mirrors M. The figure is not exactly to scale.

grating monochromator (f/4.4, 500 mm focal length, 1200 lines/mm grating). The 50 c/sec light chopper is a sectioned disk rotated by a synchronous motor. Filter F_1 is a Corning 9863 which reduces stray light from the monochromator. The monitor photocell (CsSb surface) operates as part of a Photovolt photometer, model 501-M.

The sample cuvette is a 1 mm quartz cell placed perpendicular to the excitation beam to prevent reflected light from reaching the fluorescence detector. The glass lens system L_3 is of high aperture, f/1.2. Filter combination F_2 consists of a wide band interference filter GAB K 2 and Schott filters BG 7, 1 mm, and GG 3, 2 mm. The transmission of this combination drops from a maximum value of 36 % at 450 mμ to ~9 % at 420 and 470 mμ. Schott BG 23, 2 mm is added to F_2 when the sample is illuminated with 678 mμ light. An RCA 1P21 photomultiplier serves as light detector.

Filter F_4 is water, 2 cm, to absorb heat radiation above 1.2 mμ. L_4 is a glass lens, f/2.9. For illumination of photosynthetic bacteria, F_3 is 2 × Schott RG 9, 2 mm, providing a broad transmission band with the maximum at 825 mμ, and the light source is a 150 W projection bulb. The intensity available at the sample cuvette is 12 mW/cm^2. For illumination of algae, F_3 is Schott RG 5, 2 mm and interference filter GAB 678, and the source is a 500 W bulb. In this case the intensity is 1.2 mW/cm^2. The light condenser system is that of the Aldis "Star" 5 × 5 cm slide projector minus heat filter. A rectangular stop, 10 × 30 mm, is placed in one slide of the slide transport, and a metal blank in the other. Illumination is turned on and off by means of the slide transport.

The electrical signal from the photomultiplier is amplified and rectified by a phase sensitive amplifier previously described[3]. The rectified signal is recorded by a Philips 1 second recorder. The recording apparatus responds only to the modulated fluorescence signal and rejects any signal due to scattered radiation from the photosynthetic illuminator. The filter set F_2 reduces the intensity of scattered light sufficiently to avoid noticeable increase in phototube shot noise when the cuvette is illuminated with photosynthetic light.

Over the wavelength interval 280 to 400 mμ stray light in the excitation beam is less than 1/2 %. For a typical case in which a 6 % (wet cell volume ratio) suspension of *Rhodospirillum rubrum* is excited to fluorescence, the signal from scattered stray light is estimated to be of the order of 1 % of the signal from fluorescence of bacteria, suspension medium, and cuvette over the excitation wavelength interval 280 to 380 mμ. At 390 mμ, scattered excitation light accounts for roughly 10 % of the signal, but for $\lambda \leqslant 360$ mμ the signal from scattered excitation light is less than 1 % of the fluorescence signal. The signal from fluorescence of the suspension medium and cuvette is estimated to be 10 to 20 % of the signal from bacterial fluorescence for the 335 mμ excitation maximum. Fluorescence from the medium is minimized by the use of concentrated cell suspensions.

Fortunately, the change in signal caused by photosynthetic illumination of the sample is independent of scattered light and fluorescence of the medium and cuvette. Only changes in fluorescence are recorded, and these changes can be caused only by photochemical reactions within the cells.

The intensity of the photosynthetically active illumination is determined by means of a thermopile in the sample cuvette position. Similarly the monitor phototube in the position shown in Fig. 1 is calibrated in terms of the energy intensity of the excitation beam incident at the cuvette over the entire useable wavelength interval 260 to 400 mμ. The calibration curve is converted into quantum intensity terms by the relation $I_Q = I_E/h\nu$. All excitation spectra are determined as fluorescence per unit quantum intensity of excitation.

The monochromator slits are open to a width of 3.12 mm and stopped down to an average height of 15 mm. The half-maximum-intensity bandwidth of the excitation beam is thus 5.0 mμ. The intensity of the exit slit image at the sample cuvette is attenuated by means of a small circular stop between F_1 and lens 1/2 L_1 in order to minimize photochemical reactions in the sample due to excitation light. The maximum quantum intensity of the excitation beam is obtained at about 365 mμ. At this wavelength the energy intensity averaged over the entire slit image for one chopping cycle never exceeds 0.1 mW/cm^2, which is less than 1/10 the lowest energy intensity used for photosynthetic illumination. The relative increases in fluorescence upon illumination are found to be the same whether the intensity of the excitation beam is increased or decreased by a factor of 2.

The reliability of excitation spectra obtained with the apparatus was tested by comparing the excitation spectrum of DPNH, with the absorption (1-T) spectrum as determined by means of a Zeiss PMQ II spectrophotometer. The results summarized in Table I appeared satisfactory.

TABLE I

COMPARISON OF ABSORPTION AND FLUORESCENCE EXCITATION FOR DPNH

Wavelength (mμ)	$\frac{A_\lambda}{A_{340}}$	$\frac{(f/I)_\lambda}{(f/I)_{340}}$
310	0.69	0.68
315	0.76	0.75
320	0.84	0.85
325	0.92	0.93
330	0.96	0.98
335	1.00	1.02
340	1.00	1.00
345	0.98	1.00
350	0.92	0.94
355	0.83	0.86
360	0.72	0.76
365	0.62	0.67
370	0.51	0.54
380	0.23	0.31

$\sim 4.5 \cdot 10^{-4}$ *M* DPNH in 0.05 *M* phosphate buffer pH 7.7.
$d = 1$ mm; $\Delta\lambda = 8.0$ mμ; $A_{340} = 47.5\%$. A_λ is the absorption $(1 - T_\lambda)$. $(f/I)_\lambda$ is the fluorescence per incident quantum.

MATERIALS

All organisms were grown in a light cabinet under illumination from tungsten bulbs. Photosynthetic bacteria were grown anaerobically in glass-stoppered bottles. Algae were cultured in Erlenmeyer flasks partially filled with medium through which air was bubbled continuously. Concentrated suspensions (~ 5 % wet cell volume per volume suspension) of each organism were prepared for examination.

Chromatium, strain D, was grown in a non-sterile inorganic medium containing bicarbonate, sulfide, and thiosulfate as substrates (*cf.* NEWTON AND KAMEN[4]). Bacteria were resuspended in fresh medium for observation.

Rhodospirillum rubrum, strain 4, was grown in sterile medium containing 1 % peptone (Difco) and 1/2 % NaCl in tap water. Since the peptone medium is highly fluorescent, bacteria were washed once and resuspended in a medium containing 15 m*M* Na butyrate, 1/2 % NaCl, and 10 m*M* phosphate, pH 7.0. Samples were gassed with 5 % CO_2 and 95 % N_2 before examination.

The blue alga *Anacystis nidulans* was grown in Medium C of KRATZ AND MYERS[5] initially adjusted to pH 7.4. Suspensions were examined in culture liquid.

Chlorella vulgaris was grown in an inorganic salt solution according to WARBURG[6]. The gas phase was enriched with CO_2 (5 %). The algae were examined suspended in culture liquid.

For the determination of excitation spectra for free and bound pyridine nucleotide, reduced diphosphopyridine nucleotide (Sigma) and yeast alcohol dehydrogenase (Boehringer) were used.

EXPERIMENTS

The u.v. excitation spectrum for the increase in fluorescence (420–470 mμ) caused by photosynthetic irradiation of a bacterial or algal suspension is obtained by repetition of a fixed cycle of light and dark periods. The sequence of fluorescence changes is then in general reproducible. The wavelength and intensity of the photosynthetic light remain the same for each light period, while the wavelength of the radiation for excitation of fluorescence is varied from 390 to 260 mμ in 5 mμ steps. The ratio of fluorescence signal to quantum intensity of excitation is proportional to the percentage of excitation light absorbed by fluorescent material only. Therefore the excitation spectrum indicates the absorption characteristics of the fluorescent material.

Chromatium

The sequence of fluorescence changes in *Chromatium* during and after illumination have been described in a preceding paper[2]. Upon illumination the fluorescence initially increases to a maximum and thereafter may either decrease or remain approximately constant. For determination of the excitation spectrum for the initial fluorescence increase a suspension was illuminated (12 mW/cm^2) for 3 sec every 2 min. The light interval was long enough to permit the fluorescence to reach its maximum value, and the dark interval was sufficient to allow the return to the dark steady-state level before the next light interval. The excitation spectrum (Fig. 3A) has maxima at $\sim$280 and 335 μ.

The fluorescence excitation spectrum of bacteria in the dark steady-state shows similar maxima, even though this spectrum includes fluorescence from the medium and the cuvette in addition to a contribution from scattered excitation light for $\lambda > 360$ mμ.

Rhodospirillum rubrum

A typical sequence of fluorescence changes in anaerobic *Rsp. rubrum* is shown in Fig. 2A. Illumination (12 mW/cm^2) resulted in an initial fluorescence increase followed

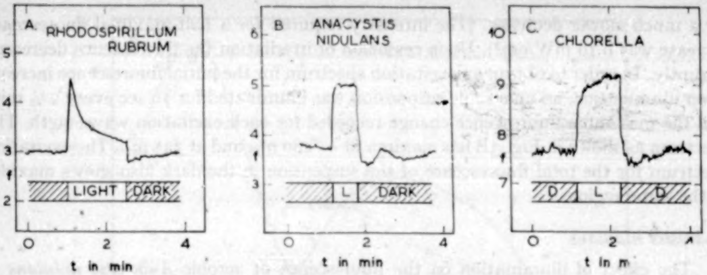

Fig. 2. Recordings of light-induced fluorescence changes in (A) anaerobic *Rsp. rubrum*, (B) aerobic *Anacystis nidulans*, and (C) aerobic *Chlorella vulgaris*.

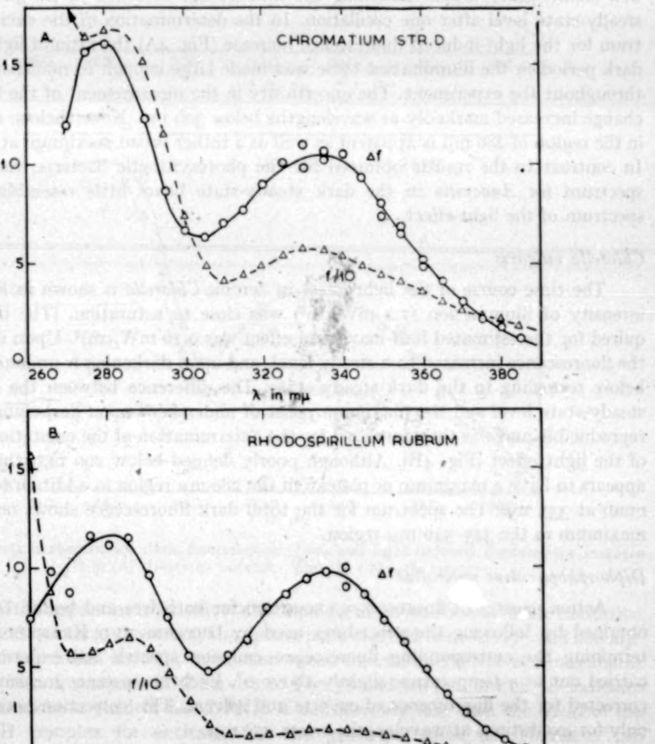

Fig. 3. Excitation spectra for dark fluorescence (*f*/10) and light-induced fluorescence increase (*Δf*) in (A) *Chromatium*, and (B) *Rsp. rubrum*.

by a much slower decrease. (The intensity required for a half-maximal fluorescence increase was 0.16 mW/cm^2). Upon cessation of irradiation the fluorescence decreased abruptly. In order to obtain an excitation spectrum for the initial fluorescence increase upon illumination, an anaerobic suspension was illuminated for 10 sec every 1 ½ min, and the maximum fluorescence change recorded for each excitation wavelength. The spectrum as shown in Fig. 3B has maxima at $\sim$ 280 mμ and at 335 mμ. The excitation spectrum for the total fluorescence of the suspension in the dark also shows maxima in the same regions.

Anacystis nidulans

The effect of illumination on the fluorescence of aerobic *Anacystis nidulans* is shown in Fig. 2B. Illumination caused an immediate increase in fluorescence to a new steady-state. Upon darkening the fluorescence returned to the previous dark steady-state level after one oscillation. In the determination of the excitation spectrum for the light-induced fluorescence increase (Fig. 4A) the ratio of light period to dark period in the illumination cycle was made large enough to maintain aerobiosis throughout the experiment. The uncertainty in the measurement of the fluorescence change increased markedly at wavelengths below 300 mμ. Nevertheless, a maximum in the region of 280 mμ is apparent as well as a rather broad maximum at $\sim$ 330 mμ. In contrast to the results obtained for the photosynthetic bacteria, the excitation spectrum for *Anacystis* in the dark steady-state bears little resemblance to the spectrum of the light effect.

Chlorella vulgaris

The time course of the light effect in aerobic *Chlorella* is shown in Fig. 2C. The intensity of illumination (1.2 mW/cm^2) was close to saturation. (The intensity required for the estimated half-maximum effect was 0.16 mW/cm)^2. Upon illumination the fluorescence increased to a steady level, and upon darkening it undershot slihgtly before returning to the dark steady state. The difference between the illuminated steady-state level and the maximum point of undershoot upon darkening was quite reproducible and was therefore used for the determination of the excitation spectrum of the light effect (Fig. 4B). Although poorly defined below 290 mμ, this spectrum appears to have a maximum or plateau in the 280 mμ region in addition to the maximum at 335 mμ. The spectrum for the total dark fluorescence shows only a broad maximum in the 325–340 mμ region.

Diphosphopyridine nucleotide

Action spectra of fluorescence excitation for both free and bound DPNH were obtained by following the procedures used by DUYSENS AND KRONENBERG[7] in determining the corresponding fluorescence emission spectra. All experiments were carried out at a temperature slightly above 0°. Each fluorescence measurement was corrected for the fluorescence of cuvette and solvent. This correction became sizable only for excitations at wavelengths below 270 mμ.

The excitation spectrum of the complex, DPNH and yeast ADH, is shown as curve 1 in Fig. 5A. Maxima occur at about 278 and 344 mμ. Addition of excess acetaldehyde causes oxidation of nearly all DPNH and a decrease in fluorescence as shown by curve 2. ADH alone (curve 3) shows only a slight fluorescence in the wavelength

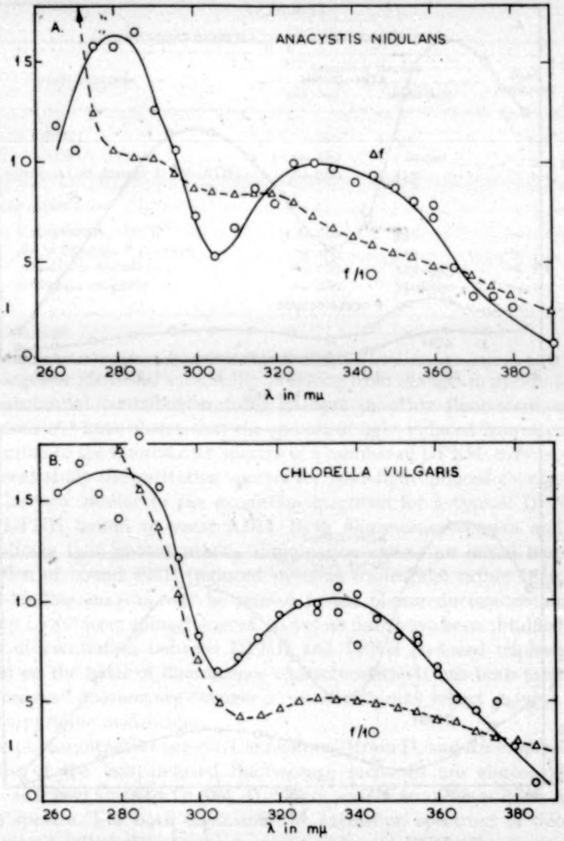

Fig. 4. Excitation spectra for dark fluorescence (*f*/10) and light-induced fluorescence increase (Δ *f*) in (A) *Anacystis nidulans*, and (B) *Chlorella vulgaris*.

region where the apparatus is sensitive. The shoulder at about 280 mμ in the excitation spectrum corresponds to the strong absorption peak of ADH between 275 and 280 mμ.

WEBER[8] has found that the excitation spectrum of free DPNH has maxima at 260 and 340 mμ. A comparison of curve 1 of Fig. 5A and curve 2 of Fig. 5B indicates that the fluorescence yield of free DPNH is considerably less than the yield of the DPNH–ADH complex for excitation in the 340 mμ region (*cf.* DUYSENS AND KRONENBERG[7]).

For comparison with the excitation spectra of fluorescence changes in photosynthetic organisms, the difference excitation spectrum, ADH–DPNH minus ADH–DPN, was obtained by subtracting curve 2 from curve 1 in Fig. 5A. The resulting

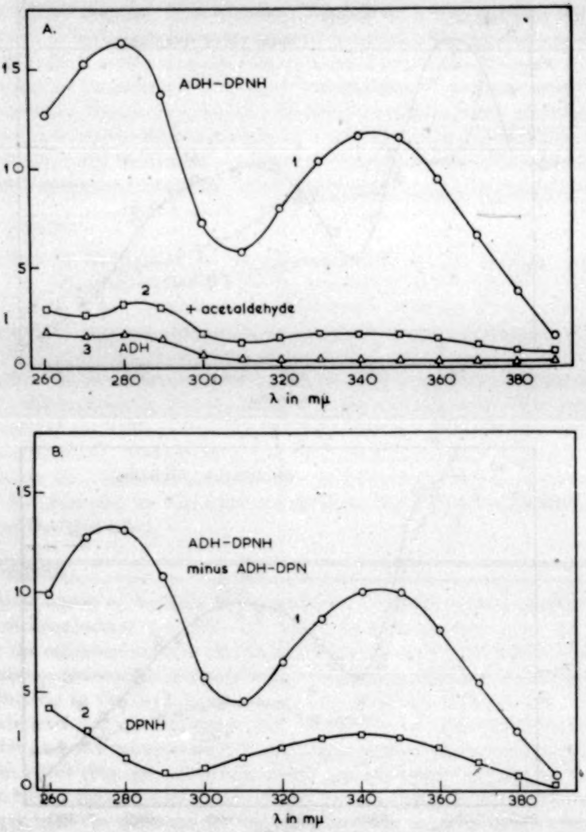

Fig. 5. Excitation spectra of fluorescence. (A) Curve 1: $1.8 \cdot 10^{-4}$ *M* DPNH, $6 \cdot 10^{-5}$ *M* yeast ADH, $2 \cdot 10^{-4}$ *M* semicarbazide, and $2.5 \cdot 10^{-2}$ *M* phosphate, pH = 7.9. Curve 2: After addition of excess acetaldehyde ($2 \cdot 10^{-2}$ *M*) to above solution. Curve 3: $6 \cdot 10^{-5}$ *M* yeast ADH. (B) Curve 1: difference spectrum upon addition of excess acetaldehyde to $1.8 \cdot 10^{-4}$ *M* DPNH and $6 \cdot 10^{-5}$ *M* yeast ADH. Curve 2: $1.8 \cdot 10^{-4}$ *M* DPNH.

spectrum (curve 1, Fig. 5B) is remarkably similar to the spectra obtained from the bacteria and algae, whereas the spectrum for free DPNH is distinctly different. These results are summarized in Table II.

DISCUSSION

The use of fluorometry as a method of following light-induced changes of intracellular pyridine nucleotide in photosynthetic organisms requires that the observed fluor-

TABLE II

CHARACTERISTICS OF EXCITATION SPECTRA

Excitation spectrum	*Location of maxima*		*Ratio first/second*
	first (mμ)	*second (mμ)*	
Free DPNH	260*	340	1.0*
Yeast ADH	~ 280	none	—
DPNH–ADH minus DPN–ADH	~ 280	345	1.3
Light effect for			
Chromstium, str. D	~ 280	335	~ 1.6
Rhodospirillum rubrum	~ 280	335	~ 1.2
Anacystis nidulans	~ 280	325–335	~ 1.6
Chlorella vulgaris	~ 280	330–340	~ 1.7

* WEBER[8].

escence changes be identified specifically as arising from changes in pyridine nucleotide without substantial contribution from changes in other fluorescent compounds. Previous studies[1, 2] have shown that the spectra of light-induced fluorescence changes are very similar to the fluorescence spectra of a number of DPNH–enzyme complexes. In the present study the excitation spectra for these light-induced changes have been shown to be very similar to the excitation spectrum for a typical DPNH–enzyme complex, DPNH bound to yeast ADH. Both fluorescence spectra and excitation spectra indicate that photosynthetic illumination causes an initial increase in the concentration of bound PNH (reduced pyridine nucleotide) rather than free PNH. The PNH-binding enzyme may be related to the photoreductase obtained by SAN PIETRO AND LANG[9] from spinach leaves. As yet no data have been obtained concerning a possible differentiation between DPNH and TPNH (reduced triphosphopyridine nucleotide) on the basis of fluorescence characteristics. It has been tacitly assumed that the observed fluorescence changes in whole cells may reflect changes in either or both of the pyridine nucleotides.

In the photosynthetic bacteria, *Chromatium*, strain D, and *Rhodospirillum rubrum* the spectra of the light-induced fluorescence increases are almost identical (*cf.* DUYSENS AND SWEEP[1] and OLSON, DUYSENS AND KRONENBERG[2]), as are also the excitation spectra. For both organisms the excitation spectrum of fluorescence in non-illuminated cells indicates the presence of bound PNH. The transient increase in bound PNH upon illumination is about 20 % for *Chromatium* and roughly 50 % for *Rsp. rubrum*. For *Chlorella* the increase appears to be 20 to 30 %.

In *Anacystis nidulans* the spectrum of the light-induced fluorescence increase differs slightly from the spectra of the bacteria in having its maximum at 460–470 mμ instead of at 440 mμ[1]. The excitation spectrum for the light effect in algae is basically similar to the spectrum for bacteria, but the 330–340 mμ peak is somewhat broader in the former case. This slight difference is not unexpected in view of the great differences in size and internal structure between the two types of organisms.

The excitation spectrum of fluorescence in non-illuminated *Chlorella* suggests the presence of PNH as does also the fluorescence spectrum determined by DUYSENS AND AMESZ[10]. For *Anacystis*, on the other hand, the corresponding excitation spectrum differs markedly from the pyridine nucleotide spectrum. This difference may be attributed to the considerable amount (up to 0.1 % dry weight) of other blue fluorescent material in *Anacystis*. FORREST, VAN BAALEN AND MYERS[11, 12] have identified the principle fluorescent substances as pteridines.

The presence of a strong peak at 280 mμ in the fluorescence excitation spectrum of the yeast ADH–DPNH complex is evidence for the transfer of quanta absorbed by aromatic amino acid residues in the protein moiety to the pyridine ring of the nucleotide moiety. Recent studies of dehydrogenases by SHIFRIN AND KAPLAN[13] indicate that pyridine nucleotides and their analogues quench protein fluorescence ($\lambda \sim 350$ mμ) in proportion to the extent of binding. Thus it appears that quanta normally appearing as protein fluorescence are transferred to nucleotide upon the formation of a protein–nucleotide complex.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation for the advise and encouragement of Dr. L. N. M. DUYSENS. Dr. J. B. THOMAS and the Biophysical Research Group at the State University at Utrecht very kindly provided laboratory space and biological material for this work. This investigation was supported by the Netherlands Organization for Pure Scientific Research (Z.W.O.).

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THE SPECIFIC REFRACTION INCREMENT OF CRYSTALLINE PROTEIN

II. α-CHYMOTRYPSINOGEN

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(Received March 25th, 1959)

SUMMARY

The refractive indices of isoionic crystals of α-chymotrypsinogen have been measured by microscope interferometry employing two procedures. In principle, the index-values are deduced from measurement of optical path of crystal relative to mother liquor by combining this value with measurements first on an air-bubble[3,4] squeezed to the same thickness as the crystal, and second, on the crystal located inside an air-bubble. The two principle refractive indices are 1.483 ± 0.003 and 1.486 ± 0.001 by method 1, and 1.480 ± 0.001 and 1.482 ± 0.001 by method 2. Separate measurements of birefringence by the polarizing microscope gave a value of 0.0039 ± 0.00005. The crystals are orthorhombic; it was not possible to measure the third refractive index. Combining these values with data[5] on the dry weight of protein per unit volume of crystal, the principal specific refraction increments are on average 0.00192 and 0.00195. These values are in fairly good agreement with the value of 0.00185 for dilute solutions of α-chymotrypsinogen.

INTRODUCTION

In a previous communication[1] data were presented on the specific refraction increments of two crystalline β-lactoglobulins. Further measurements are reported here on the specific refraction increment of crystalline α-chymotrypsinogen. The specific refraction increment of a salt-free crystal may be defined as its refractive index minus that of water divided by the concentration of dry protein per unit volume of crystal (g/100 ml). The parameter is usually determined in dilute solutions (about 1 g/100 ml) and the aim of the investigation was to compare this known value with that obtained for crystalline protein. Data on the optical properties of crystalline proteins are of general interest in that they may provide information on crystal structure and they are also of interest in cell interferometry. This is a method[2] for assaying the dry mass or protein content of single biological cells.

As in the previous study[1] the refractive indices of the protein crystals have been

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measured by a method introduced by AMBROSE[3] and by KLUG AND WALKER[4]. Measurements have also been made by a simpler alternative variation of the method which suggested itself to us. The basic idea is to determine the optical path in the crystal relative to mother liquor and air by means of an interferometer microscope. The refractive indices of the latter media are known and it is shown that the index of the crystal can be simply calculated. The specific refraction increments of α-chymotrypsinogen are obtained by combining the index-values with data on the concentration of protein in the crystal given by BLUHM AND KENDREW[5].

METHODS

Salt-free (isoionic) crystals of α-chymotrypsinogen were kindly prepared for us by Dr. J. KRAUT. They were obtained by passing a solution of α-chymotrypsinogen through an amberlite exchange resin column, the upper part of which consisted of a mixture of IR 120-H$^+$ and IRA 400-OH$^-$ and the lower part of a layer of IR 120-H$^+$. The ion-free solution from the column crystallized in a tabular form (Fig. 1). According to X-ray diffraction observations[5,6] the crystals are orthorhombic and are flattened along the c-axis. They showed dome (011), (101) and a pyramidal face which, according to rough measurements of angle coupled with the unit cell measurements was (171).

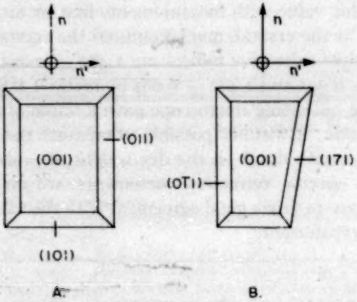

Fig. 1. Shows the habit of the crystals of α-chymotrypsinogen. The arrows indicate the direction of the electric vector for which the two refractive indices n and n^1 were measured.

Well-formed crystals of suitable size (thickness 10 to 30 μ) were chosen under a binocular microscope from the stock preparation, and transferred in their mother liquor in a small-volume pipette to a glass slide previously ringed with vaseline. By expelling air from the pipette numerous air-bubbles were formed in the mother liquor; a coverslip (No. 2) sealed off the preparation (Fig. 4). The general principles underlying the two methods of measuring the refractive indices of the crystals are as follows.

Method 1

The preparation was mounted in a special device, constructed along lines similar to the one first made by Dr. P. M. B. WALKER (Fig. 2). In this device the coverslip and slide could be pressed together in a controlled fashion so as to squash the air-bubble and render its thickness equal to that of the crystal (Fig. 3). During squashing, a series of readings of optical path difference (o.p.d.) relative to mother liquor were made through the crystal (Δ_{cm}) and air-bubble (Δ_{lm}). As the air-bubble was deformed so that its thickness approached that of the crystal, the value of the ratio of Δ_{cm}/Δ_{lm}

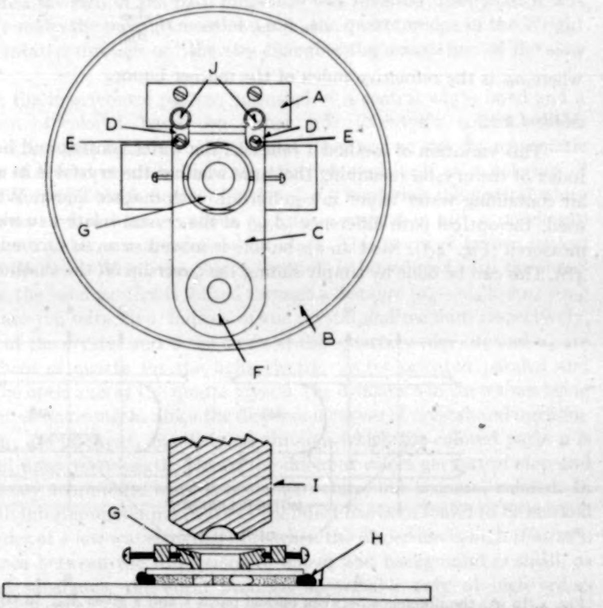

Fig. 2. Schematic of device used for squeezing the air-bubble. A block (A) screwed on to a rotary stage (B) is connected (screws at E, other connection not shown) to a plate (C) by means of two leaf springs (D). The springs can be distorted by screws (J) so as to level the plate. An annular ring (G) is pivoted in the plate (C) and is in contact with the specimen (H). I is the microscope objective. The height of the plate above the specimen, and hence the degree of squash, is controlled by the adjustment screw (F).

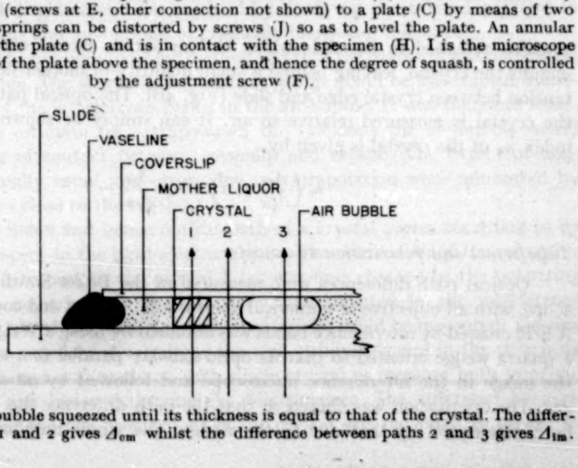

Fig. 3. Shows the air-bubble squeezed until its thickness is equal to that of the crystal. The difference in optical paths 1 and 2 gives Δ_{cm} whilst the difference between paths 2 and 3 gives Δ_{lm}.

approached a maximum which was not affected by a slight deformation of the crystal; the latter condition was indicated by stress lines in its surface.

It can simply be shown that that refractive index n_c of the crystal is given by

$$n_c = n_m + (n_m - 1)\,\frac{\Delta_{cm}}{\Delta_{lm}} \qquad (1)$$

where n_m is the refractive index of the mother liquor.

Method 2

This variation of method 1 relies on the water content and hence the refractive index of the crystal remaining the same whether the crystal is in mother liquor or in air containing water vapor in equilibrium with mother liquor. When this method is used, the optical path difference (Δ_{cm}) of the crystal relative to mother liquor is first measured (Fig. 4A). Next an air-bubble is moved so as to surround the crystal (Fig. 4B). This can be done by simply sliding the coverslip on the vaseline ring. The crystal remains attached to the slide whilst the air-bubble moves with the coverslip and engulfs the crystal, leaving behind a small quantity of mother liquor held by surface tension between crystal edge and slide (Fig. 4B). The optical path difference (Δ_{cl}) of the crystal is measured relative to air. It can simply be shown that the refractive index n_c of the crystal is given by

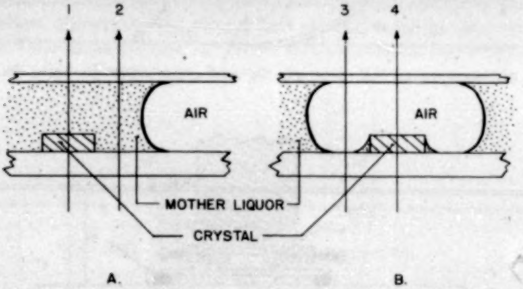

Fig. 4. In (A) the difference between optical paths 1 and 2 gives Δ_{cm}. In (B) the air-bubble has been moved to surround the crystal; the difference between paths 3 and 4 gives Δ_{cl}.

$$n_c\left(\frac{\Delta_{cl}}{\Delta_{cm}} - 1\right) = n_m\,\frac{\Delta_{cl}}{\Delta_{cm}} - 1 \qquad (2)$$

Interference and polarization microscopy

Optical path differences were measured in the Baker-Smith interference microscope, with an objective of numerical aperture (N.A.) 0.25 and condenser N.A. < 0.1. A field crossed by interference bands was obtained by using a Wright-eyepiece, that is, a quartz wedge oriented so that its optic axis lay parallel to a vibration direction in the image in the interference microscope and followed by an analyser oriented at 45°. The orientation was chosen so that the path difference due to the object could be compensated by that due to the wedge. The whole number of wavelengths in the value of optical path difference was estimated in the usual way in white light (tungsten), while the fractional part was estimated to the nearest tenth by eye in monochromatic ($\lambda = 546$ mμ) light isolated from a mercury arc by filters. When an air-bubble was measured the sign of the path difference was reversed from what it was with the crystal. To make the measurement of o.p.d., the quartz wedge in the Wright eyepiece could be rotated through 90° thereby changing the orientation of the slow and fast axes.

In white light the interference pattern consisted of a central white band and a symmetrical system of colored bands on either side (Newton's colors). When estimating the o.p.d. in white light it was noted that there was no strictly achromatic band in the image of crystal or air bubble; the effect showed itself as a coloring of the previous approximately-black bands of order ± 0.5 bordering the central white band. At high orders of interference (about 30 λ for a crystal in air) a previously colored band (order 1.5) became approximately black. This is a well known optical effect and arises as follows. When the object (crystal) is introduced into the interference microscope, the band pattern is shifted through a distance $(n_c - n_m)d/\theta(n_e - n_o)$ where n_c and n_m are the refractive indices of the crystal and medium respectively, d is the thickness of the crystal and θ the angle of the quartz wedge. n_o and n_e are the refractive indices of quartz for the light electric vector oriented parallel and perpendicular to the optic axis of the quartz crystal, the difference in the values being nearly independent of wavelength. Since the dispersion curves of crystal and medium, or medium and air, are different, the distance through which the colored pattern is shifted will depend upon wavelength. Hence the different colors get out of step and the central previously achromatic band is no longer white, but becomes colored. In measurements with interferometer microscopes the effect has been found to be marked in thin objects (order of a few wavelengths) only when the dispersion is high (FAUST[8]). When the difference between the dispersions of object and background is small, as in most biological substance, the effect becomes appreciable only at high orders (15 to 30) of interference. Any uncertainty as to which was the correct band to be used for measuring band displacement was resolved by studying the colored patterns in the images of crystals and air-bubbles of gradually increasing thickness.

A difficulty in the bubble squashing method was that the coverslip was not always parallel with the slide. The shape of the resulting wedge could be ascertained by observing the shape of the interference bands in the air-bubble. It was therefore sometimes necessary to estimate by extrapolation the thickness the air-bubble would have at the region of contact between coverslip and crystal. The extent of non-parallelism was usually small and errors due to extrapolation were minimized by choosing air-bubbles close to the crystal.

The refractive index and hence optical path of a crystal varies according to its orientation with respect to the light electric vector. The orientation of the refractive index ellipsoid with respect to the crystal faces was first checked in the polarizing microscope. These orthorhombic crystals were then oriented in the interference microscope so that one of the principal refractive indices could be measured. The one being measured was noted by determining the sign of birefringence in the polarizing microscope. No attempt was made on each single crystal to measure both principal refractive indices, and hence determine the birefringence. The difference between the optical paths for the two orientations of the crystal was too small to permit an accurate determination in the interference microscope. Instead, birefringence was measured separately on thick crystals in the polarizing microscope.

Birefringence was measured in the usual way in the polarizing microscope with a Wright-eyepiece, the quartz wedge having a scale marked on it. If t is the geometrical thickness of the crystal, the retardation τ measured on the polarizing microscope is given by $\delta n.\, t$, where δn is the birefringence. The value of t is obtained by measuring the optical path difference (Δ_{cm}) in the interference microscope with the crystal oriented so that the value of the refractive index n_c is available from the previous experiments. Then

$$t = \Delta_{cm}/(n_c - n_m) \tag{3}$$

and the birefringence δn is given by

$$\delta n = \frac{\Delta_{cm}}{\tau} \cdot (n_c - n_m) \tag{4}$$

The refractive index of the mother liquor was measured on an Abbé refractometer. All measurements were made at a temperature close to 27°.

RESULTS AND DISCUSSION

The methods described here fore measuring the thickness, refractive index and birefringence of protein crystals probably represent, in certain respects, an advance on previous methods. Previously it was necessary first to free the crystal from mother liquor and then to immerse it in media of gradually increasing refractive index until a match was obtained as judged, for example, by the BECKE line method. Such treatment may alter the refractive index. However, the present methods are not applicable to crystal fragments of irregular shape.

The experimentally determined ratios of optical difference are given in Tables I and II, where the primed symbols indicate that the light electric vector is parallel

TABLE I

VALUES OF Δ_{cl}/Δ_{lm} FOR α-CHYMOTRYPSINOGEN CRYSTALS*

No.	Δ_{cm} in λ	Δ'_{cm} in λ	$\frac{\Delta_{cm}}{\Delta_{im}}$	$\frac{\Delta'_{cm}}{\Delta'_{im}}$
36	11.5		0.446	
42		13.9		0.458
43		8.8		0.465
47		7.4		0.465
51	2.85		0.475	
52		8.9		0.453
55		13.5		0.446
56		11.2		0.461
57		7.3		0.443
58	4.1		0.467	
60		5.5		0.447
62	16.1		0.436	
63	9.1		0.436	
64	6.95		0.425	
		Average:	0.446 ± 0.008	0.455 ± 0.003

* Obtained by method 1.

TABLE II

VALUES OF Δ_{ci}/Δ_{cm} FOR α-CHYMOTRYPSINOGEN CRYSTALS*

No.	Δ_{cm} in λ	Δ'_{cm} in λ	$\frac{\Delta_{ci}}{\Delta_{cm}}$	$\frac{\Delta'_{ci}}{\Delta'_{cm}}$
1		6.8		3.20
3		6.45		3.27
4		6.5		3.34
5		6.6		3.28
7		3.1		3.29
8	4.8		3.32	
9	10.3		3.21	
10	2.5		3.22	
12	2.35		3.36	
13	2.3		3.39	
15	6.6		3.29	
17		5.8		3.17
18	4.65		3.25	
19		2.95		3.22
21	2.45		3.27	
22		3.0		3.30
23	3.0		3.33	
24		2.5		3.20
25	3.2		3.28	
26		3.5		3.20
27	4.95		3.33	
28		3.0		3.31
29	2.35		3.30	
30	2.80		3.21	
32		2.85		3.15
		Average:	3.29 ± 0.016	3.24 ± 0.019

* Obtained by method 2.

to the short (slow) axis of the crystal (Fig. 1). The salt-free mother liquor had a refractive index of 1.3343, corresponding to a protein concentration of about 1%. The calculated values for refractive index of the crystal are, by method 1 1.483 ± 0.003 (n) and 1.486 ± 0.001 (n^1), and by method 2 1.480 ± 0.001 (n) and 1.482 ± 0.001 (n^1).

The birefringence determined in the polarizing microscope was the same for all crystals and had a mean value of 0.0039 ± 0.00005 (Table III). The values of birefringence obtained from interference microscope measurements were 0.003 (method 1) and 0.002 (method 2). It has already been pointed out that the accuracy of these latter results cannot be high since they are obtained by subtraction of two measurements.

On account of crystal shape it was not possible to obtain an accurate value of the refractive index for light vibrating parallel to the c-axis. However, observations of the sign of birefringence of crystals turned on their edge and allowed to dry in air showed this refractive index to be lower than n^1. A rough measurement on one crystal indicated its magnitude to be similar to n. Previous observations upon crystals dried from the mother liquor while on a slide had indicated that birefringence was approximately maintained upon drying.

The aim of this study was to calculate the specific refraction increments of crystalline α-chymotrypsinogen. The concentration of dry substance per unit volume of

TABLE III

VALUES OF BIREFRINGENCE δn FOR CRYSTALS OF α-CHYMOTRYPSINOGEN

No.	τ *in* λ	$n^1 - n = \delta n$
1	0.168	0.0037
2	0.158	0.0036
8	0.278	0.0039
9	0.268	0.0038
11	0.413	0.0040
19	0.074	0.0037
31	0.306	0.0041
35	0.407	0.0042
36	0.337	0.0040
38	0.348	0.0040
41	0.493	0.0040
45	0.208	0.0038
46	0.346	0.0039
	Average:	0.0039 ± 0.00005

TABLE IV

SUMMARY OF RESULTS

Parameter	*Method 1*	*Method 2*	*Average of 1,2*	*Polarizing Microscope*
n	1.483 ± 0.003	1.480 ± 0.001	1.482	
n^1	1.486 ± 0.001	1.482 ± 0.001	1.484	
δn	0.003	0.002	0.0025	0.0039 ± 0.00005
a	0.00194 ± 0.00003	0.00190 ± 0.00001	0.00192	
a^1	0.00197 ± 0.00001	0.00192 ± 0.00001	0.00195	

crystal from the experimental data in BLUHM AND KENDREW[5] is given by the product of crystal density (1.215) and fraction of dry protein by weight in the crystal (0.642). Hence the concentration is 78.0 g/100 ml. The values of specific refraction increment obtained (Table IV) by the two methods differ by only 2%. The average values for the two specific refraction increments, corresponding to the two principal refractive indices measured, are 0.00192 and 0.00195, which values are to be compared with that of 0.00185 obtained in dilute solution by WILCOX, KRAUT, WADE AND NEURATH[9].

When electrolytes such as salts and amino acids crystallize there is a large decrease in the specific refraction increments. For example, in the case of sodium chloride the decrease is about 50% and is presumably due to changes partly in the partial specific volumes and partly in the polarizability of the constituents. Apparently such changes are small in the case of protein molecules and our experiments show that the proportionality between refractive index and concentration does not differ much between dilute solutions and crystals of α-chymotrypsinogen. Some variation is of course to be expected, due to alignment of protein molecules resulting in birefringence. In contrast to the results obtained upon simple salts there is, in fact, a small increase in the refraction increment of α-chymotrypsinogen, amounting to about 3.5% on average. This increase is similar to those obtained previously[1] with the monoclinic and orthorhombic forms of β-lactoglobulin, namely 3.5% and 7% respectively. Whether this small apparent increase is due to a systematic error in the experiments or has a real physical basis is not yet known.

ACKNOWLEDGEMENTS

It is a pleasure to record appreciation of the kind hospitality of Professor H. S. BENNETT, who also provided facilities for this work. We are also much indebted to Dr. J. KRAUT for providing us with crystals of α-chymotrypsinogen and to Dr. J. C. KENDREW for drawing our attention to these crystals.

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Biochim. Biophys. Acta, 37 (1960) 25–33

THE EFFECT OF SOME PURINE DEOXYRIBOSIDES ON THE INCORPORATION OF [^{14}C]FORMATE AND [^{32}P]ORTHOPHOSPHATE INTO DNA OF ASCITES TUMOR CELLS *IN VITRO*

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(Received April 1st, 1959)

SUMMARY

It has been found that under various experimental conditions deoxyadenosine, but not deoxyguanosine or deoxyinosine, causes pronounced and consistent inhibition of the incorporation of the carbon of formate into DNA–TMP of Ehrlich ascites tumor cells *in vitro*. This effect could be reversed by simultaneous addition of deoxyguanosine. Deoxyguanosine was found to have the same action in experiments on the incorporation of [^{32}P]labelled orthophosphate into DNA.

Experiments with [^{14}C]formate did not show any consistent inhibitory effect of deoxyadenosine on the labelling of DNA–TMP in Yoshida ascites tumor cells.

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The following abbreviations are used in the text: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; TMP, thymidine-5′-phosphate; dAMP, deoxyadenosine-5′-phosphate; dCMP, deoxycytidine-5′-phosphate; dGMP, deoxyguanosine-5′-phosphate.

INTRODUCTION

Previous experiments[1,2] with isotopically labelled compounds suggested that deoxyadenosine inhibits DNA synthesis in Ehrlich ascites tumor cells *in vitro*. These investigations have now been extended to include experiments on the effect of other purine deoxyribosides on the incorporation of [^{14}C]formate and [^{32}P]orthophosphate into DNA of both the Ehrlich and Yoshida strains of ascites tumor cells.

MATERIALS AND METHODS

The procedures and experimental conditions were in general the same as those described by Klenow[1], unless otherwise indicated. The incubation time was three hours in all experiments.

RESULTS

Addition of deoxyadenosine to suspensions of Ehrlich ascites tumor cells was found to cause consistent and pronounced inhibition of incorporation of [^{14}C]formate into

TABLE I

[^{14}C] FORMATE INCORPORATION INTO THE DNA OF EHRLICH ASCITES CELLS

Experiment	*Deoxyribotide isolated*	*Addition*				
		None counts/min/µmole	*Deoxyadenosine* % of control	*Deoxyguanosine* % of control	*Deoxyinosine* % of control	*Deoxyadenosine plus deoxyguanosine* % of control
4.7 µmoles deoxycytidine/ml	dGMP	11,900	(3)	16	10	—
3.5 µmoles purine	dAMP	7.540	(2)	66	13	—
deoxyriboside/ml	TMP	55.400	8	135	99	—
4.1 µmoles deoxycytidine/ml	dGMP	3.420	(3)	15	—	(4)
3.1 µmoles purine	dAMP	2,200	(3)	105	—	(4)
deoxyriboside/ml	TMP	17.150	12	235	—	201
4.9 µmoles deoxycytidine/ml	dGMP	4.300	(6)	22	—	—
3.7 µmoles purine	dAMP	3,000	(6)	101	—	—
deoxyriboside/ml	TMP	31.300	4	132	—	—
	dGMP	1,780	(4)	(6)	—	(2)
3.4 µmoles purine	dAMP	1,280	(4)	44	—	(2)
deoxyriboside/ml	TMP	4.950	7	121	—	108
	dGMP	1,020	(11)	(9)	(16)	—
3.9 µmoles purine	dAMP	688	(8)	(36)	(21)	—
deoxyriboside/ml	TMP	4,080	6	71	124	—
2.4 µmoles dCMP/ml	dGMP	5,680	20	15	15	—
3.5 µmoles purine	dAMP	3,980	13	25	16	—
deoxyriboside/ml	TMP	11,830	15	35	122	—
2.1 µmoles dCMP/ml	dGMP	3.070	(4)	25	—	(7)
3.1 µmoles purine	dAMP	1,980	(2)	—	—	(5)
deoxyriboside/ml	TMP	22,800	10	162	—	103

The incubation media contained 500 mg cells (wet wt.) in a total volume of 2.1–2.4 ml. The figures in parentheses are derived from counts less than 50% above background.

DNA–TMP, both in the presence and in the absence of added deoxycytidine or dCMP. This incorporation was, however, with two exceptions, stimulated by the addition of deoxyguanosine, while addition of deoxyionosine had no effect or a small stimulating effect. Usually less [^{14}C] formate was incorporated into DNA–TMP in the cells of the control flasks when deoxycytidine was omitted from the incubation media, or when deoxycytidylic acid was substituted (Table I).

When both deoxyadenosine and deoxyguanosine were added to Ehrlich cell suspensions incubated with [^{14}C]formate, the inhibitory effect of deoxyadenosine was substantially reversed independently of the presence of deoxycytidine or dCMP (Table I).

This effect of deoxyguanosine was confirmed and examined in more detail by measuring [^{32}P]orthophosphate incorporation into DNA with constant deoxyadenosine concentrations and increasing deoxyguanosine concentrations (Fig. 1). In the experiment illustrated it is seen that in the absence of added deoxyguanosine the [^{32}P]incorporation into DNA with deoxyadenosine (4 μmoles/ml) was about 20% of the control value without deoxyadenosine. Increasing concentrations of deoxyguanosine progressively reversed this inhibition. About 2 μmoles of deoxyguanosine per ml completely overcame the deoxyadenosine effect, so that the incorporation of [^{32}P]phosphate into DNA was the same with or without deoxyadenosine added. At still higher concentrations of deoxyguanosine, the deoxyadenosine effect was reversed, and a small stimulating effect was observed. In the absence

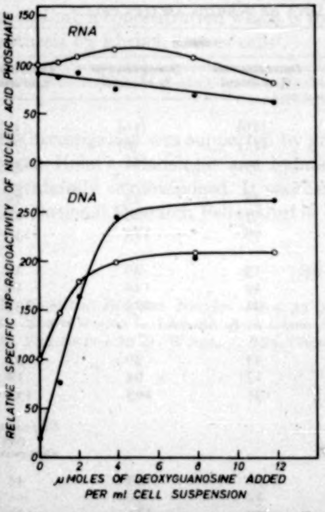

Fig. 1. Variation of incorporation of $^{32}PO_4^{\equiv}$ with concentration of deoxyguanosine. Incubation for 3 h in the presence of 4.2 μmoles deoxycytidine per ml, and 500 mg cells (wet wt.). Final volume 2.4 ml. ○, No deoxyadenosine added. ●, 3.2 μmoles of deoxyadenosine added per ml.

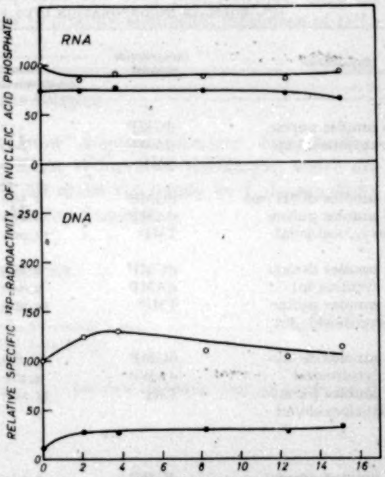

Fig. 2. Variation of incorporation of $^{32}PO_4^{\equiv}$ with concentration of guanosine. Experimental conditions as for Fig. 1. ○, No deoxyadenosine added. ●, 3.0 μmoles of deoxyadenosine added per ml.

of added deoxyadenosine increasing concentrations of deoxyguanosine stimulated incorporation of ^{32}P into DNA. Concentrations of about 4 µmoles/ml or greater increased the incorporation by a factor of approximately 2. Deoxyguanosine had only a small effect on incorporation of ^{32}P into RNA. In the presence of deoxyadenosine it caused slight inhibition, which increased with increasing concentrations of deoxyguanosine.

The effect of deoxyguanosine cannot be reproduced with guanosine. Two µmoles of guanosine approximately doubled [^{32}P]phosphate incorporation into DNA in the presence of deoxyadenosine, but increase of the guanosine to as much as 15 µmoles/ml had little further stimulating effect (Fig. 2). In the absence of added deoxyadenosine, guanosine at concentrations of 2–5 µmoles/ml had a slight stimulating effect on [^{32}P]incorporation into DNA. Guanosine had little or no effect on [^{32}P]incorporation into RNA.

In suspensions of Yoshida ascites tumor cells of rats, deoxyadenosine failed to elicit the same marked inhibition of [^{14}C]formate incorporation into DNA–TMP observed with the cells of the Ehrlich strain. The effect of deoxyadenosine ranged from a 50% inhibition to a 32% augmentation of [^{14}C]formate incorporation into DNA–TMP (Table II). Both deoxyinosine and deoxyguanosine (with a single exception) stimulated the [^{14}C]formate incorporation into DNA–TMP. In the case of the Yoshida cells the purine deoxyribosides appeared generally to reduce incorporation

TABLE II

[^{14}C]FORMATE INCORPORATION INTO THE DNA OF YOSHIDA ASCITES CELLS

Experiment	*Deoxyribotide isolated*	*Addition*			
		None counts/min/µmole	*Deoxyadenosine % of control*	*Deoxyguanosine % of control*	*Deoxyinosine % of control*
3.9 µmoles purine deoxyriboside/ml	dGMP	577	(26)	(16)	(31)
	dAMP	805	(11)	(6)	(71)
	TMP	3,510	50	91	148
2.4 µmoles dCMP/ml 3.5 µmoles purine deoxyriboside/ml	dGMP	1,560	38	25	70
	dAMP	2,230	31	26	28
	TMP	11,000	76	113	155
4.7 µmoles deoxycytidine/ml 3.5 µmoles purine deoxyriboside/ml	dGMP	2,190	13	20	12
	dAMP	2,840	19	120	16
	TMP	14,900	92	223	138
4.7 µmoles deoxycytidine/ml* 3.5 µmoles purine deoxyriboside/ml	dGMP	5,260	43	39	30
	dAMP	4,480	17	94	19
	TMP	31,200	131	203	132
					Deoxyadenosine plus deoxyguanosine
4.1 µmoles deoxycytidine/ml 3.1 µmoles purine deoxyriboside/ml	dGMP	1,243	56	33	44
	dAMP	2,100	22	96	—
	TMP	17,300	132	181	174

500 mg cells in a total volume of 2.1–2.4 ml. The figures in parentheses are derived from counts less than 50% above the background.

* Purification of the deoxyribotides may have been inadequate in this experiment since only column and charcoal purification methods were used.

of [^{14}C]formate into the DNA deoxyguanylic and deoxyadenylic acids to a lesser extent. The relative incorporation of [^{14}C]formate into the DNA purine deoxyribotides also differs in the two strains of cells. The average ratio of the specific activities of deoxyguanylic and deoxyadenylic acid is only about 0.7 in Yoshida cells, as compared to 1.2 to 1.5 in Ehrlich cells (Table I and KLENOW[1], Tables I and IV).

DISCUSSION

The constant pronounced inhibitory effect of deoxyadenosine on reactions involved in DNA synthesis seems to be specific for this compound. As appears from this communication and from a previous one[1], adenine, adenosine, adenosine plus guanosine, guanosine, deoxyribose, deoxyguanosine and deoxyinosine do not have this effect.

The observation that deoxyguanosine, but not guanosine, overcomes the inhibition effect on Ehrlich ascites tumor cells, suggests that deoxyadenosine inhibits DNA synthesis by interfering directly or indirectly with synthesis of deoxyguanosine or a closely related precursor of DNA.

The finding that deoxyadenosine fails to inhibit DNA synthesis by Yoshida ascites tumor cells *in vitro* suggests that the inhibition observed with Ehrlich cells is not a universal phenomenon. In the study on DNA synthesis in rabbit thymus nuclei carried out by FRIEDKIN AND WOOD[3], deoxyadenosine was also found to have no inhibitory effect. The deoxyadenosine concentration used was, however, only 0.1 µmoles/ml, a concentration which is too low to produce significant inhibition of DNA synthesis by Ehrlich ascites cells[1].

ACKNOWLEDGEMENTS

This investigation was supported by grants from "Augustinusfonden", Tømrermester Jørgen Holm's Mindelegat and Fabrikant Einar Willumsens Mindelegat, which are all gratefully acknowledged. It was carried out under the tenure by L. LANGER of a U.S. National Research Fellowship in the Medical Sciences.

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CHROMATOGRAPHIC ISOLATIONS OF PIG AND HUMAN MELANOCYTE-STIMULATING HORMONES

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(Received March 25th, 1959)

SUMMARY

Pig β-melanocyte stimulating hormone and a human melanocyte stimulating hormone have been isolated by chromatography on a sulphonic acid resin. Both require to have many of their basic groups discharged by a high pH, and the human melanocyte stimulating hormone also requires a hydrogen-bonding agent in the solution, if the partition of the active substances is not to be overwhelmingly in favour of the resin. Cations seem relatively ineffective in displacing these substances from the resin.

INTRODUCTION

Methods of counter-current distribution[1-3] and electrophoresis[1,4,5] have been used for the separation of β-melanocyte-stimulating hormone from the other substances present in oxycellulose concentrates of partially purified pituitary extracts. Ion-exchange chromatography has been highly successful[6] with other peptides of similar molecular size and therefore seemed worth investigating for this purpose.

Pig β-melanocyte-stimulating hormone

The starting material used was a by-product in the isolation of corticotropin by the method of DIXON AND STACK-DUNNE[7]: the unretarded fraction on chromatography on a carboxylic ion-exchange resin of an oxycellulose concentrate[8] of an acid-acetone extract[9] of pig pituitary glands. Frozen glands were used in place of acetone-dried glands so as to recover more corticotropin in the A_1 form instead of as A_2[7]. Otherwise the oxycellulose concentrate was prepared as previously described[7], except that the proportion of oxycellulose used was doubled. From 9,234 glands (2,4 kg wet wt) 960 mg concentrate was obtained. The concentrate was chromatographed on a carboxylic ion-exchange resin equilibrated and developed with sodium phosphate buffer, and from 400 mg concentrate 180–190 mg corticotropin A_1 and 30 mg of an unretarded fraction were obtained. Desalting, here and in the work described below, was performed by adsorbing the material from an acidified solution onto a carboxylic resin in the free acid form, washing out the salts with 5 % acetic acid and displacing the polypeptides with 50 % acetic acid[10]. The unretarded fraction possessed MSH activity in the intact frog. This unretarded fraction served as a

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Abbreviation: MSH, melanocyte-stimulating hormone.

starting material for the isolation of β-MSH although it is now known that a large part of the melanocyte-stimulating activity of the glands is lost in making the oxycellulose concentrate by this method, and indeed the α-MSH is probably completely lost (*cf.* LEE[5]).

The conditions used by HIRS, STEIN AND MOORE[6] to elute the most strongly adsorbed peptides that they studied did not show any displacement of the melanocyte-stimulating activity of this fraction from a sulphonated polystyrene resin (Zeo-Karb 225, 1 % cross-linked, 200–400 mesh), nor did a solution containing 1 *M* Ba^{++} at pH 8 (*cf.* CRAMPTON, MOORE AND STEIN[11]). Since SAMSONOV AND FADDEEVA[12] had found that insulin was only eluted from a sulphonic resin of low cross-linking when the pH was high, a solution with pH 10 containing 0.2 *M* ethylene diamine and 0.1 *M* acetic acid was tried and proved suitable for eluting MSH. FASELLA, BAGLIONI AND TURANO[13] pointed out the versatility of this buffer system, but it should be noted that, despite their claim, the ethylene diamine and acetic acid are not appreciably volatile when neutralized by each other. Fig. 1 shows a chromatogram of the fraction described above. The large retarded peak gave on desalting 6 mg β-MSH (*i.e.* 1.6 mg/1000 glands). The amino acid analysis of an acid hydrolysate was: Met 1.00; Ser 0.98, Tyr 0.90, Phe 0.99, Asp 1.86, Glu 2.17, Gly 2.02, Pro 3.08, Ala 0.20, Val 0.14, Leu 0.14 moles on adjusting the total of the first 8 amino acids to 13 moles. The analysis was made by the method of MOORE, SPACKMAN AND STEIN[14] without analysing for bases.

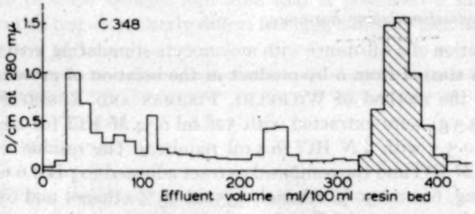

Fig. 1. Chromatogram of 30 mg β-MSH concentrate on sulphonated polystyrene resin Zeo-Karb 225 1 % cross-linked 200–400 mesh. Column 38.5 cm × 0.68 cm^2, equilibrated and developed with a solution containing 0.2 *M* ethylenediamine, 0.1 *M* acetic acid and 0.03 % toluene. Shaded area re-run on C355 (Fig. 2a).

The presence of the alanine, valine and leucine could be due to (a) unrelated impurities not removed by chromatography, (b) impurities related to β-MSH in that they could have similar structure, or (c) contamination of the sample after chromatography. HARRIS[15] discussed the presence of such amino acids in MSH preparations.

The flow rate was limited by the fact that the resin was compressed by applied pressure, and the column took 3 days to run. With lower loads than that shown in Fig. 2a yields of only 40–50 % were obtained on re-running. Use of a resin of 2 % cross-linker but otherwise identical allowed much faster running and gave a good recovery on re-running (Fig. 2b) so this resin is recommended (Zeo-Karb 225; 2 % cross-linked; 200–400 mesh).

The inactive peak observed (as in Fig. 1) in many samples and running with 0.6 of the retardation of the hormone was treated in 33-ml effluent with 2 ml thioglycollic acid at 80° for 19 h and it acquired melanocyte-stimulating activity and emerged on re-running at the position of β-MSH (*cf.* corticotropin[16–18]).

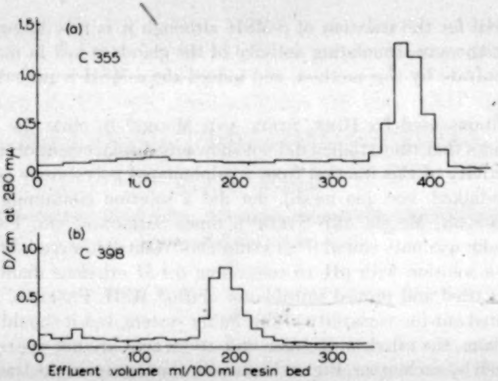

Fig. 2. Rechromatograms of β-MSH. Columns equilibrated and developed with a solution of 0.2 *M* ethylenediamine, 0.1 *M* acetic acid and 0.03 % toluene. (a) Column of 37.3 cm × 0.68 cm. Zeo-Karb 225 1 % cross-linked 200–400 mesh. Recovery in peak based on absorbancy: 76 %. Flow rate 2.0 ml/h. (b) Column of 26.9 cm × 1.1 cm diameter. Zeo-Karb 225 2 % cross-linked 200–400 mesh. Tubes for collecting effluent each contained 1 ml 5 % acetic acid, so absorbancy was read after this dilution. Recovery in peak based on absorbancy: 73 %. Flow rate 4.7 ml/h.

Human melanocyte-stimulating hormone

The preparation of a substance with melanocyte-stimulating activity from human pituitary glands started from a by-product in the isolation of growth hormone by a modification of the method of WILHELMI, FISHMAN AND RUSSELL[19]. 516 acetone-dried glands (53.5 g) were extracted with 528 ml 0.34 *M* KCl for 1 h, the pH being kept adjusted to 5.5 with 2 *N* HCl (6.5 ml required). The residue was re-extracted with 528 ml 0.3 *M* KCl and the combined extract adjusted to pH 8.6 with 2 *N* NaOH. After centrifuging, 600 ml 50 % ethanol, 155 ml 96 % ethanol and 650 ml saturated ammonium sulphate were added and precipitates removed at various stages, the whole operation being performed at 0–4°. The final supernatant was then discarded from the growth-hormone preparation and was available as a starting material for the isolation of MSH.

The solution was acidified to pH 3.4 with 1 ml 10 *N* HCl, 20 ml acetic acid and 2 ml thioglycollic acid. A precipitate formed which was difficult to remove by filtration until it had been increased and coagulated by heating to 80° in 30 min and maintaining the temperature for 5–10 min before cooling. The filtrate was "desalted"[10] by passing through a column 3 cm × 7 cm diameter of polymethacrylic acid resin (Amberlite IRC-50, XE-64) in the free acid form, washing the salts out of the resin with 5 % acetic acid and displacing the product with 50 % acetic acid. The final solution was dried while frozen (yield, 1.13 g).

Of this product 622 mg were extracted 3 times with 0.1 *N* acetic acid and treated with an equal weight of oxycellulose in solution at a concentration of 1 mg/ml in 0.1 *N* acetic acid. The adsorbed material was eluted with 0.1 *N* HCl, most of the HCl removed by ion exchange with the acetate of Amberlite IR4B and the product dried while frozen (yield, 132 mg). This is essentially the method of ASTWOOD, RABEN, PAYNE AND GRADY[8].

The product was chromatographed on a carboxylic resin by the method of Dixon and Stack-Dunne[7] so as to recover in the retarded fractions some corticotropic activity. The unretarded fraction contained most of the melanocyte-stimulating activity and was "desalted" (53 mg).

Samples of the first dry product described above had been used to find conditions suitable for desorbing the human MSH activity from the sulphonated polystyrene resin. The solution of 0.2 *M* ethylene diamine and 0.1 *M* acetic acid suitable for the chromatography of pig β-MSH did not give measurable desorption, nor did the following solutions: 2.5 *M* $CaCl_2$– 0.2 *M* ethylenediamine (pH 10); 1 *M* barium acetate– 0.2 *M* ethylenediamine– 0.1 *M* acetic acid (pH 9.9); 4 *M* NaCl– 0.2 *M* ethylenediamine– 0.1 *M* acetic acid. Solutions of guanidine hydrochloride (*cf.* Luck *et al.*[20]) and urea at pH 10 gave measurable desorption of the active material from the resin, and proved suitable for chromatography. 1.2 *M* urea displaced the MSH more completely from the resin than did 2.75 *M* guanidine hydrochloride.

The unretarded fraction from the carboxylic column (53 mg) was therefore chromatographed with urea in the buffer solution and Fig. 3 shows the result. From the large retarded peak active material was isolated (6.3 mg, 0.02 mg/gland or 0.2 mg/g dry glands). This material moved as a single band (as revealed by the ninhydrin and bromophenol blue reagents) when it was submitted to high voltage ionophoresis on paper in pyridine–acetic acid buffers of pH values 3.5 and 6.4. Its electrophoretic mobility in both of these systems indicated that it possessed a slightly basic iso-electric point. In this respect it clearly differs from pig β-MSH which has an iso-electric point between 5 and 6[3,4].

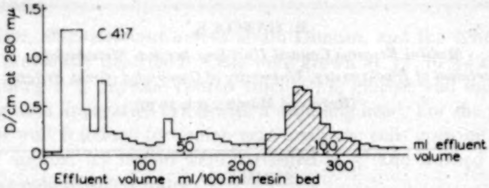

Fig. 3. Chromatogram of human MSH concentrate. Column of 35.0 cm × 1.00 cm^2 Zeo-Karb 225, 2% cross-linked, 200–400 mesh, equilibrated and developed with a solution of 1.2 *M* urea, 0.2 *M* ethylenediamine, 0.1 *M* acetic acid and 0.03% toluene. Flow rate 5.4 ml/h. The tubes for collecting the effluent each contained 1 ml 5% acetic acid for 3 ml effluent, so the absorbancy is that observed after this neutralization. The shaded area shows the effluent desalted to yield 6.3 mg material which possessed MSH activity in the whole frog at a dose of 5 mμg.

ACKNOWLEDGEMENTS

I am greatly indebted to Miss M. B. Thomas for her skilled assistance throughout the work. I am most grateful to Professor F. G. Young, Dr. A. Korner and Mr. B. R. Slater for the human material discarded in the preparation of growth hormone and to the many pathologists who co-operated with the Medical Research Council in collecting the human glands. I also thank Mr. M. W. Rees and Miss B. Worboys for the amino acid analysis, Dr. J. I. Harris, Dr. J.-P. Waller and Mr. M. W. Rees for much helpful discussion and the Medical Research Council for a grant.

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THE AMINO ACID COMPOSITION OF THE PROTEIN AND CELL WALL OF *STAPHYLOCOCCUS AUREUS*

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(Received March 23rd, 1959)

SUMMARY

The amino acid composition of the cell protein and cell wall of *S. aureus* (strain Duncan) have been determined quantitatively; the rates of incorporation of amino acids into these cell fractions during growth can be calculated from these data. Only 32% of the weight of the cell wall from exponentially-growing organisms can be accounted for as amino acids and amino sugars.

INTRODUCTION

Amino acids occur in a number of separable fractions of bacterial cells; among these the amino acid pool, the cell protein and the cell wall have been investigated in some detail in certain organisms. Quantitative analyses of the amino acid pool of *Staphylococcus aureus* (strain Duncan) during exponential growth have been reported[1], and, to provide data with which to compare these results, it was of interest to determine the amino acid composition of the cell protein.

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Abbreviation: TCA = Trichloracetic acid.

Since the term "protein", when referring to fractions of bacterial cells, is defined to a considerable extent by the method of isolation, any analysis of a "protein" fraction is strictly valid only for fractions prepared by the specified method. The hot-TCA-insoluble fraction, prepared by procedures derived from that of SCHNEIDER[2] has often been regarded as a homogeneous protein fraction. It is now recognised that in *S. aureus* this fraction contains considerable quantities of cell wall material[3,4]; it also contains any ethanol-extractable "proteins", which should perhaps be regarded as a distinct and separate fraction as they have been in *Escherichia coli*[5]. The procedure used here has been to determine the composition of both the hot-TCA-insoluble fraction of the cell, and also of cell walls prepared by a conventional method. The former fraction includes the cell wall peptides, which undergo only a very small amount of degradation when the TCA treatment is reduced to a minimum (PARK AND HANCOCK, in preparation). The fraction whose composition is the difference between these two is regarded here as constituting the cell protein; it includes therefore any ethanol-soluble compounds, which may form a distinct sub-fraction.

The rates of incorporation of individual amino acids into the protein and cell wall during growth can be calculated from these data, and, since this organism derives the majority of its amino acids from the external medium, the rates of entry of amino acids into the cell can also be obtained.

MATERIALS AND METHODS

Organism and media

The organism, *Staphylococcus aureus* strain Duncan, and the synthetic medium (S) were those previously described[1]. Cells were grown at 37° in 3-l cultures in PG medium (containing 2% peptone (Bacto Difco), 2% glucose and inorganic salts[6]), using a rotating flask apparatus[7] fitted with a sampling head. For the preparation of protein and cell wall fractions for amino acid analyses, cells growing exponentially at a density of approx. 0.3 mg/ml were harvested at 5° and washed twice by centrifugation in ice-cold distilled water.

Growth was followed by measuring the culture O.D. at 700 mμ in the Beckman DU spectrophotometer; the relation between O.D. and culture density (dry wt. cells/ml) was constant during the exponential phase of growth, and the mean generation time (the time for doubling of bacterial mass) was calculated from the rate of increase of O.D.

Preparation of fractions for analysis

To prepare the combined (protein + cell wall) fraction, washed cells were extracted with 5% (w/v) TCA at 90° for 6 min; the residual fraction was washed twice in ice-cold 5% TCA. Centrifugations were carried out at 4000 × *g* for 15 min at room temperature.

Cell walls were prepared by disintegrating washed cells in the Mickle disintegrator, following the procedure used by CUMMINS AND HARRIS[8] but with omission of ribonuclease treatment. Before the first separation of the crude cell wall sediment, all operations were carried out at 5°; at this stage the preparation was suspended in distilled water and heated for 10 min in a boiling water bath to inactivate any autolytic systems present (*cf.*[9]).

Each fraction was hydrolysed under reflux in 6 *N* HCl–100 % formic acid (1 : 1) for 24 h; acid was removed at 45° under an air stream, and the sample was re-dried 3 times from small volumes of water.

Amino acid analysis

The hydrolysate of the combined (protein + cell wall) fraction was separated on a single column of Dowex 50 × 4 (150 × 0.9 cm) following the procedure of MOORE AND STEIN[10], and 2-ml fractions were collected. The cell wall preparation was divided into 2 parts which were analysed on 2 separate columns of Amberlite IR-120 as used in the more recent method of MOORE, SPACKMAN AND STEIN[11]. The ninhydrin colour values in effluent peaks have been expressed as μmoles amino acid by reference to a leucine standard.

The dry wt. of washed cells, or of the appropriate cell fraction, used for each analysis was determined from the mean weight of triplicate samples after drying at 105° in an air oven for 8 h.

RESULTS

The amino acid composition of the fractions analysed is presented in Table I.

In the preparation analysed in Table I the weight of combined (cell wall + protein) fraction recovered was equivalent to 655 mg/g cells. The composition of the protein, obtained indirectly by subtracting the quantity of each amino acid in the

TABLE I

AMINO ACID COMPOSITION OF SOME FRACTIONS OF *S. aureus*

These analyses did not include tryptophan and cyst(e)ine.

Amino acid	*μmole amino acid/g cells in*			
	Protein + cell wall	*Cell wall*	*Protein*	*Pool**
Glycine	1178	183	995	2.8
Alanine	865	132	733	8.1
Glutamic acid	743	42	701	39.6
Aspartic acid	565	2.3	563	38.0
Lysine	551	48	503	2.2
Leucine	359	3.7	355	2.6
Valine	310	3.5	307	1.9
Serine	299	6.6	292	3.4
Threonine	275	1.0	274	1.0
Isoleucine	270	2.8	263	8.5
Arginine	183	—**	183	2.2
Phenylalanine	174	—	174	1.3
Proline	148	—	148	16.8
Tyrosine	112	—	112	2.4
Histidine	84	—	84	1.7
Methionine	78	1.2	77	6.7
NH_3	686	128	558	—

* Taken from [1].
** Not detectable.

cell wall fraction from that in the combined fraction, is included in Table I. In the combined fraction 89 % of the ninhydrin-reacting material loaded onto the column was recovered as amino acids and ammonia; cyst(e)ine and tryptophan were not recovered quantitatively and not estimated in the eluate. In this fraction, small unidentified peaks were eluted at 125 ml and at 220 ml; these contained, respectively, the equivalent of 21 and 53 μmoles amino acid/g cells.

It was not easy to obtain a completely quantitative recovery of the cell wall fraction, and under the conditions used here 150–180 mg of cell wall were obtained from 1 g of exponentially-growing cells on different occasions; the cell wall composition in Table I has been expressed on the basis of a mean value of 165 mg wall/g cells. The 4 amino acids alanine, glycine, glutamic acid and lysine account for 95 % of the total quantity of amino acids in the cell wall fraction. The other amino acids are present in only trace amounts, but relative to the valine, leucine or methionine content, the quantity of serine present in the wall is about double that expected from contamination with protein of composition similar to the average value for the cell protein. In separate experiments glucosamine and muramic acid were found to undergo breakdown (varying from 60 % to 100 %) under the conditions of hydrolysis used, so no quantitative estimations of these components of the wall were attempted in these experiments. One unidentified component, containing the equivalent of 13.4 μmoles amino acid/g cells, was eluted before lysine from the column used to analyse the basic amino acids; it is possible that this was a breakdown product of the amino sugars.

It is apparent from these results that the amino acids together account for only approx. 24 % of the weight of the cell wall; if the hexosamine content is added to this figure (using a ratio hexosamine/glutamic acid of 1.8:1[4]), only 32 % of the weight can be accounted for by the hexosamine-peptide polymer. Since the cell walls of *S. aureus* strain H contain ribitol phosphate (teichoic acid)[12], it appeared possible that this material might also occur in the walls of strain Duncan and account for some or all of the remaining 68 % of the weight. Hydrolysates (1 h, 1 *N* HCl, 100°) of cell walls of strain Duncan contain components corresponding chromatographically with ribitol and ribitol-5-phosphate, and in hydrolysates of ^{32}P-labelled walls ^{32}P appears in the position of the latter compound. No glycerol is detectable, however (compare MITCHELL AND MOYLE[13]).

For comparison with the amino acid composition of the cell protein, an amino acid analysis of the pool of cells growing exponentially under the same conditions, but in synthetic medium, is included in Table I (from [1]).

From these results, the rate of incorporation of any amino acid into the cell protein or cell wall during growth can be calculated. For a culture with a mean generation time of 50 min, the value usually obtained in these experiments, the rate of incorporation of each amino acid (in μmoles/g cells/h) into the appropriate cell fraction is given by the quantity in Table I, multiplied by 1.2.

DISCUSSION

An interesting feature of the composition of the cell protein of *S. aureus* is the high content of the acidic amino acids, which together account for nearly 25% of the total residues; the quantity of ammonia recovered suggests that a considerable amount of these acidic residues could have been present originally as the amides. The relative amounts of the amino acids in the protein of *S. aureus* are not unlike those in the protein of *E. coli*[5], except that glycine is present in much larger amounts in *S. aureus*. There appears to be no correlation between the relative quantities of amino acids in the protein and those which are maintained in the amino acid pool during exponential growth; a tentative hypothesis to explain this situation has been put forward previously[1].

It is interesting that, of the 5 amino acids which are most abundant in the cell protein, 4 (glutamic acid, lysine, alanine and glycine) are those which constitute the cell wall hexosamine-peptide component; their relative quantities in the 2 fractions are in almost similar sequence. It is perhaps unlikely that this preponderance in both cellular fractions is a consequence of a common step in the biosynthesis of both cell wall and protein, since, in *S. aureus* H at least, most of the glutamic acid and some of the alanine in the cell wall are in the D-configuration[14], but it is interesting to speculate on possible causes for this situation. The ratio of the 4 major amino acids in the wall of *S. aureus* Duncan is 1 glutamic acid: 1.1 lysine: 3.1 alanine: 4.6 glycine, compared with the ration 1: 1.2: 3.5: 3.8 found in the wall of *S. aureus* H[14].

It is hoped that these data will be of use in studies of the kinetics of amino acid uptake and protein synthesis in *S. aureus*, and some of them have been used in this way in the following paper.

ACKNOWLEDGEMENTS

I am very grateful for the help and advice of Dr. M. W. REES and Miss B. WARBOYS, whose co-operation made it possible to carry out the amino acid analyses. These investigations were carried out during the tenure of a Medical Research Council Scholarship.

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ACCUMULATION OF POOL AMINO ACIDS IN *STAPHYLOCOCCUS AUREUS* FOLLOWING INHIBITION OF PROTEIN SYNTHESIS

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(Received March 23rd, 1959)

SUMMARY

Determinations have been made of the quantities of individual amino acids in the pool of *S. aureus* (strain Duncan) during exponential growth. The pool levels of the twelve amino acids studied (which constitute 70% of the total amino acid pool) remain approximately constant during growth.

When protein synthesis in growing cells is blocked by chloramphenicol, there is a rapid accumulation of most amino acids in the pool. The measured rates of accumulation of glutamic acid, aspartic acid and proline are close to the rates at which these amino acids were being incorporated into cell protein when chloramphenicol was added; the relevance of these results to the role of the pool amino acids in the synthesis of protein is discussed.

Addition of *p*-fluorophenylalanine produces a similar increase in the total amino acid pool; it has been found that this analogue is incorporated into protein, synthesis of which continues at a reduced rate.

INTRODUCTION

This paper describes the effects on the components of the amino acid pool in growing cells of *Staphylococcus aureus* (strain Duncan) of two substances, chloramphenicol and *p*-fluorophenylalanine, which inhibit the synthesis of protein in this organism. This investigation was carried out with the hope that the effects of these inhibitors would provide information concerning the role of the pool amino acids in the synthesis of cell protein.

Chloramphenicol, at concentrations above 10 μg/ml, inhibits almost completely the synthesis of[1], and incorporation of amino acids into[2,3], cellular protein, and inhibits the synthesis of certain enzymes[4,5], in both intact and disrupted cells of *S. aureus*. The locus of the inhibitory action of this antibiotic has been shown to be an intermediate step in the process of protein synthesis[6]. In intact cells of *S. aureus* the amino acid analogue *p*-fluorophenylalanine inhibits enzyme synthesis[4], and the related substance *p*-chlorophenylalanine inhibits protein synthesis and incorporation of [^{14}C]phenylalanine into protein[7]. *p*-Fluorophenylalanine can be incorporated into the proteins of a number of micro-organisms[8–10], and its growth-inhibitory action has been ascribed to the synthesis of such abnormal proteins rather than to inhibition of protein synthesis *per se*[11].

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Abbreviations: CAP = chloramphenicol; PFP = *p*-fluorophenylalanine; TCA = trichloracetic acid; DMF = N,N-dimethylformamide.

MATERIALS AND METHODS

Organism and conditions of growth

The organism, *Staphylococcus aureus* strain Duncan, the media, and the conditions and measurement of growth were the same as in the previous paper except that for experiments with PFP, the synthetic medium S was used[12]. For these experiments and for those with [^{14}C]proline, the cultures were grown in 100-ml quantities of medium in shake flasks. For extraction of the pool amino acids, samples of the culture were taken into centrifuge tubes cooled in an ice–NaCl mixture; after about 2 min the cooled sample was centrifuged at 3000 × *g* for 10 min at room temperature, and the cells were washed twice in ice-cold distilled water by similar centrifugations.

CAP and PFP were added to cultures when the bacterial density reached 0.3–0.4 mg/ml.

Extraction of pool amino acids

For estimations of total pool amino acids the cells were extracted with 5 % TCA for 15 min at 5°; the extract was shaken 3 times with ether to remove TCA and an aliquot (corresponding normally to approx. 1 mg cells) was taken for amino acid estimation. For separation by paper chromatography, the pool amino acids were extracted with N,N-dimethylformamide as described previously[12]; after removal of lipids the extract was concentrated under an air stream at 45° to a volume suitable for application to paper chromatograms (approx. 10 µl). Satisfactory separation was obtained when fractions from not more than 10 mg cells were used, containing approx. 2–3 µmoles total amino acid.

Paper chromatography

The technique and solvent systems used for separating amino acids have been described previously[12]. The method does not completely resolve the amino acids leucine/isoleucine/phenylalanine or methionine/valine. The minor components of the pool (serine, threonine, cyst(e)ine, tyrosine, tryptophan and arginine) are present in such small amounts in normal cells that they could be estimated only if papers were overloaded with the other major components, and under these conditions separations were impaired. Consequently, estimations were made of only glutamic and aspartic acids, proline, glycine, alanine, histidine, lysine, valine/methionine and leucine/isoleucine/phenylalanine: these 12 amino acids constituted approx. 70 % of the total pool in normal cells under the conditions used here.

For quantitative amino acid estimations, papers were sprayed with the ninhydrin detection spray of Connell *et al.*[13] to reveal the positions of amino acids, then flooded with methanolic borate and dried in an air stream to remove ammonia. The appropriate area of the paper was then cut into small pieces, which were immersed in the ninhydrin reagent for quantitative estimation. Known amounts (0.1–0.5 μmole) of alanine were chromatographed under the same conditions and used as standards.

Amino acids (except proline) were estimated by the ninhydrin method of COCKING AND YEMM[14]; 1 *M* acetate buffer pH 4.7 was used in place of citrate buffer. Glutamic acid was used as a standard.

Proline was estimated by the method of TROLL AND LINDSLEY[15]. In the present experiments the amount of lysine in the pool was similar to that of proline, and ornithine was not detectable on paper chromatograms; the preliminary removal of these amino acids was not therefore carried out, since their colour yields are only approx. 15 % and 10 %, respectively, of that of proline by this method[16].

For detection of *p*-fluorophenylalanine, the cold-TCA-insoluble fraction of the cells was hydrolysed in 6 *N* HCl at 105° for 16 h in a sealed tube, and acid was removed by drying under an air stream at 45°. The hydrolysed material was dissolved in 5 ml of water and shaken for 1 h with washed charcoal; after washing the charcoal with water by filtration, the aromatic amino acids were eluted by shaking with 5 % phenol in 20 % acetic acid[17] for 1 h, and the eluate was concentrated *in vacuo* and re-dried from small volumes of water. *p*-Fluorophenylalanine was separated from the other aromatic amino acids in the methyl ethyl ketone–*tert.*-butanol–water (2:2:1) solvent of BOISSONAS[18]: adequate resolution was obtained in a single dimension, and the use of a second solvent was found unnecessary.

Membrane filters were used as described previously[12]. For determining the total radioactivity in the cells, a sample of the culture was filtered rapidly and the cells on the filter washed twice with ice-cold water. The radioactivity in the protein fraction was determined by immediately pipetting a second identical sample into an equal volume of ice-cold 10 % TCA; after 30 min at 5°, the TCA-insoluble material was filtered and washed twice with 5 % TCA containing 1 mg of the appropriate non-radioactive amino acid/ml. Control experiments using samples prepared by centrifugation in the usual manner showed that such filtration gives complete recovery both of whole cells and of the cold-TCA-insoluble fraction.

The radioactivity in the pool was calculated as the difference between the radioactivity in these two fractions.

For radioactivity determinations the filters were mounted in aluminium planchettes, and held flat by tightly-fitting wire rings during drying under a lamp. Radioactivity was determined using a G-M tube and scaler in the conventional manner; at least 1000 counts were recorded on each sample.

The *dry weight* of cells used for each analysis was determined by drying duplicate samples in an air oven at 105° for 8 h.

Chloramphenicol was obtained from Parke, Davis & Co., DL-*p*-fluorophenylalanine from Hoffmann-La Roche, and generally-labelled [^{14}C]proline from the Radiochemical Centre, Amersham, Great Britain.

RESULTS

Pool amino acids during normal growth

As a preliminary to investigating changes in the pool after inhibition of protein synthesis, the levels of pool amino acids during normal exponential growth were determined. It was found that there is little change in the levels of those amino acids which were estimated (Fig. 1), except in the case of alanine; this is in accord with the observation that the total amino acid pool of *S. aureus* remains approx. constant during growth under these conditions[12]. Consequently, if uptake of an amino acid into the pool forms an intermediate step in the synthesis of cell material, the rate of utilisation for such synthesis must be approximately equal to the rate of uptake into the pool.

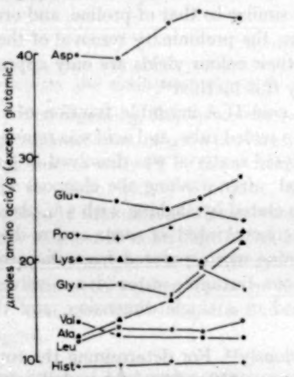

Fig. 1. Quantities of individual amino acids in the pool during exponential growth in PG medium, determined after separation by paper chromatography except proline, which was determined directly. The results for glutamic and aspartic acids were obtained in a different experiment from those for the other amino acids.

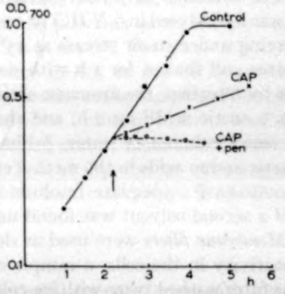

Fig. 2. Changes in culture optical density (700 mμ) following addition of chloramphenicol (50 μg/ml), with and without simultaneous addition of penicillin (0.6 μg/ml) during exponential growth in PG medium. Additions made at time indicated by arrow.

Effects of CAP on pool size

When CAP (50 μg/ml) is added to an exponentially-growing culture of *S. aureus* under the conditions used here, the rate of increase of O.D. falls by approx. 80 % (Fig. 2). Hot-TCA-insoluble nitrogen continues to increase, although utilisation of amino acids for protein synthesis is inhibited by 90 % or more; the increase is due almost entirely to synthesis of the hexosamine-peptide component of the cell wall[8]. The residual O.D. increase in the presence of CAP can be almost completely abolished by addition of penicillin (0.6 μg/ml) (Fig. 2); this result is in accord with observations that penicillin inhibits the synthesis of cell wall material[19].

Following addition of CAP there are immediate and rapid increases in the total amino acid pool and in the concentrations of most individual amino acids (Fig. 3). The increases in pool levels of some amino acids were too small for accurate measurements of rates to be made by this method. Attempts made to measure rates for these amino acids by the addition of [^{14}C]amino acid simultaneously with CAP showed that for alanine, isoleucine, leucine and valine the increases in pool level were complete in 5 min or less; measurement of accumulation rates for these amino acids was not therefore possible by this technique, although it is certain that rapid increases in pool size occur.

The rates of accumulation in the pool of glutamic and aspartic acids and proline (taken from the data in Fig. 3) are very close to the rates of incorporation of each amino acid into cell protein at the time of addition of CAP (calculated from the amino acid analysis in the previous paper, and the culture density and mean generation time at the time of CAP addition) (Table I).

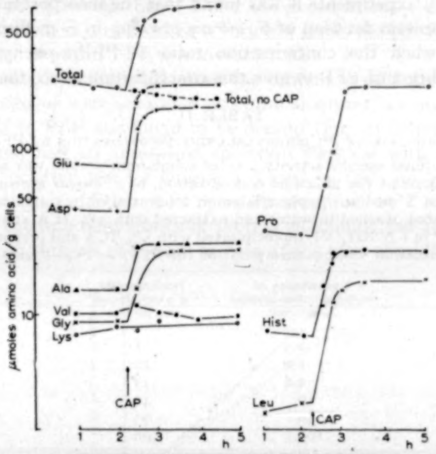

Fig. 3. Changes in the total amino acid pool and in individual components after addition of chloramphenicol (50 μg/ml) during growth in PG medium. Glutamic and aspartic acids were estimated in a different experiment from the other amino acids; proline was estimated separately. Chloramphenicol was added at the time indicated by the arrow, when the bacterial density was 0.32 mg/ml.

TABLE I

ACCUMULATION OF POOL AMINO ACIDS FOLLOWING ADDITION OF CHLORAMPHENICOL

The rates of uptake in the first column are derived from the mean generation time (75 min in this experiment) at the time of addition of CAP (50 μg/ml) and the amino acid composition of the cell protein (previous paper). Those in the second column are from the data in Fig. 3.

	Rate (μmoles/g cells/h) of	
	Uptake into protein at time of CAP addition	*Accumulation in pool after addition of CAP*
Aspartic acid	452	400
Glutamic acid	561	560
Proline	118	150

More accurate timing of samples can be achieved using membrane filters to separate cells from culture medium, and this method was used to measure the effect of addition of CAP on the rates of uptake of $[^{14}C]$proline into the pool and protein fractions during exponential growth. It was found (Fig. 4) that no detectable change occurs in the rate of total uptake of proline into (pool + protein) on addition of CAP; however, the proline which enters the cell accumulates in the pool instead of being incorporated into protein. (This experiment was carried out in medium containing

0.5 M NaCl; under these conditions the amount of proline in the pool is increased to a level similar to the amount in the cell protein, enabling more accurate estimation of the two fractions—unpublished results of the author.)

Effects of PFP on pool size

In preliminary experiments it was found that the incorporation of [^{14}C]phenylalanine into the protein fraction of *S. aureus* growing in S medium is almost completely inhibited when the concentration ratio DL-PFP/L-phenylalanine is 100:1 (Table II). On addition of PFP to give this concentration ratio, the total amino acid

TABLE II

INHIBITION OF [^{14}C]PHENYLALANINE INCORPORATION BY PFP

L-[^{14}C]phenylalanine (final specific activity $1.2 \cdot 10^4$ counts/min/µmole) was added, together with DL *p*-fluorophenylalanine at the indicated concentration, to *S. aureus* growing exponentially at approx. 0.3 mg/ml in S medium (L-phenylalanine concentration 0.5 µmoles/ml). After 60 min the cells were harvested, washed in water and extracted with 5% TCA at 100° for 15 min; the residue was dissolved in 1 N NH_4OH, reprecipitated with 5% TCA and prepared for radioactivity determination. Control incorporation (no PFP) = 1840 counts/min.

Concentration of DL-p-fluorophenylalanine (µmoles/ml)	*Inhibition (%) of phenylalanine incorporation*
0.5	5
1.0	10
2.0	13
10	40
50	95
100	98

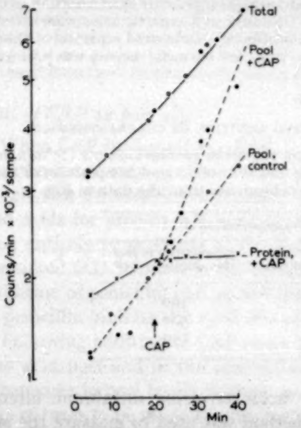

Fig. 4. Uptake of [^{14}C]proline into protein and pool during growth in PG medium containing [^{14}C]proline (0.1 µC/ml) and 0.5 M NaCl. Duplicate 4-ml samples were taken onto membrane filters and into TCA. Chloramphenicol was added to one culture (●—●) when the bacterial density reached 0.35 mg/ml.

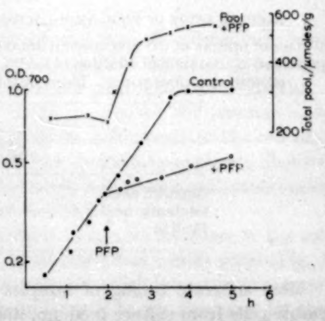

Fig. 5. Changes in culture optical density and total amino acid pool (TCA-extracted) following addition of *p*-fluorophenylalanine (50 µmoles/ml) during growth in S medium (phenylalanine concentration 0.5 µmoles/ml). Addition made at time indicated by arrow.

pool increases in a manner similar to that following the addition of CAP (Fig. 5), and paper chromatography shows that the levels of most amino acids increase; the culture O.D. continues to increase at approx. 25 % of the previous rate. When the rate of accumulation of proline in the pool was measured it was found to be lower than that following the addition of CAP (Fig. 6), suggesting that some continued utilisation of proline might be occurring. Since under these conditions the incorporation of phenylalanine into protein is reduced to a very low level, the possibility that protein is synthesised which contains PFP was investigated.

When the aromatic amino acids from the protein of cells grown for 1 h in the presence of the analogue were separated on chromatograms, a component chromatographically similar to PFP was found to be present (Fig. 7). After elution from the paper, this material had an absorption spectrum identical with that of authentic PFP, and gave a positive test for fluorine[20]. Based on the rate of accumulation of proline in the pool after addition of PFP, which is approx. 25 % less than that after addition of CAP, the rate of synthesis of protein containing the analogue is approx. 25 % of the rate of synthesis of normal protein at the time of addition.

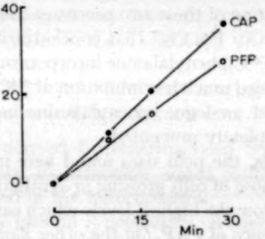

Fig. 6. Rates of accumulation of proline in the pool (TCA-extracted) after addition of *p*-fluorophenylalanine (50 μmoles/ml) and chloramphenicol (50 μg/ml) during growth in S medium.

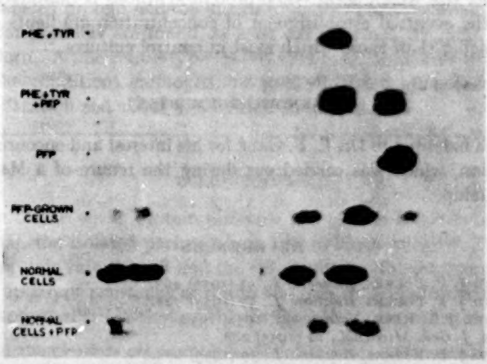

Fig. 7. Descending chromatogram of the aromatic amino acids from protein hydrolysates after growth for 60 min in S medium with and without *p*-fluorophenylalanine (50 μmoles/ml). As a control, a sample of normal cells was fractionated immediately after addition of *p*-fluorophenylalanine.

DISCUSSION

The accumulation of amino acids in the pool of *S. aureus* when protein synthesis is inhibited, either partially by PFP or completely by CAP, is compatible with a process in which the amino acids of the pool are biosynthetic precursors of cell protein. Following the addition of CAP, the rates of accumulation of the three amino acids which form major components of the pool are close to those which would be expected for precursors of the inhibited reaction of protein synthesis. However, these results do not constitute unequivocal evidence for such a process, since similar results might be obtained if both protein and pool amino acids were derived from a common precursor, when blocking of the reaction leading to protein could result in an increase in the rate of the alternative reaction, pool formation.

p-Fluorophenylalanine has now been shown to be incorporated into the protein of a number of micro-organisms[8,9] and it would seem probable that *p*-chlorophenylalanine could be incorporated into protein in a similar manner. Synthesis of cell wall (which in *S. aureus* contains D-glutamic acid and no phenylalanine) continues in the presence of high concentrations of *p*-fluorophenylalanine (unpublished observations of the author). A combination of these two processes could account, at least partially, for the findings of GALE AND FOLKES[7] that concentrations of *p*-chlorophenylalanine which completely inhibit [^{14}C]phenylalanine incorporation into the hot-TCA-insoluble fraction of *S. aureus* produced much less inhibition of [^{14}C]glutamic acid incorporation, although at high ratios of analogue : phenylalanine increases in hot-TCA-insoluble L-glutamic acid were completely prevented.

For most amino acids, the pool sizes found here in cells growing in a complex medium are larger than those of cells growing in synthetic medium[12] but they appear to be still considerably below the highest levels which can be maintained, for example those reached in the presence of CAP. On the other hand the very small increases in pool size of some amino acids (lysine, methionine, valine) induced by CAP suggest that the normal pool level of these amino acids may be the maximum possible. The increases in pool size which occur when protein synthesis is inhibited by CAP must also result in the eventual establishment of concentration gradients of amino acids considerably higher than those which exist in control cultures.

ACKNOWLEDGEMENTS

I am very much indebted to Dr. E. F. GALE for his interest and encouragement during this investigation, which was carried out during the tenure of a Medical Research Council Scholarship.

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THE REQUIREMENTS FOR SERUM ALBUMIN METABOLISM IN SUBCELLULAR FRACTIONS OF LIVER AND BRAIN

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(Received March 17th, 1959)

SUMMARY

1. Particulate fractions derived by centrifugation from both liver and brain homogenates have been shown to catabolize added serum albumin. The enzymic activity appears to be localized in the mitochondria.

2. The degradation of the protein is dependent on energy-yielding processes and is markedly stimulated by added ATP and CoA. The process therefore, does not appear to be a simple hydrolysis.

3. A major percentage of the products obtained from the breakdown of [^{14}C]-phenylalanine labeled protein may be identified as free phenylalanine.

4. Additional products are found, at least one of which contains phenylalanine in a combined form. A nucleotide-type spectrum is associated with this material.

5. Under the conditions employed, 15–30 % of added albumin is metabolized by the liver preparation and about 3 % by that of the brain.

INTRODUCTION

The metabolism of the serum protein molecule has been extensively investigated in the intact animal, the isolated perfused liver and in tissue slices[1–9]. The mechanism of entry of the protein into the cell and the subsequent metabolic events are oscbure. Reports on the apparent resistance of serum albumin to degradation by homgenate fractions have appeared[10, 11], although the more highly organized systems are known

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The following abbreviations are used: PCA, perchloric acid; ATP, adenosine triphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; CoA, coenzyme A; GSH, reduced glutathione; DPN, DPNH, diphosphopyridine nucleotide and its reduced form respectively; TPN, triphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid.

to catabolize this protein. This communication deals with the properties of a subcellular particulate fraction obtained from both rat liver and brain which is capable of metabolizing rat serum albumin at physiological pH.

METHODS

Preparation of particulate fractions

Male albino rats of the Holtzman strain, weighing 150–200 g were used. All preparative procedures were conducted in the cold. The animals were sacrificed by exsanguination and the livers and brains rapidly removed and minced. The liver tissue was homogenized in the Potter-Elvejhem homogenizer machined to give a 0.1-mm clearance between mortar and pestle[12]. Brain tissue was homogenized in an all-glass Dounce-homogenizer (obtained from Blaessig Co., Rochester, N.Y.). The homogenization medium used initially for the liver was composed of sucrose 0.126 *M*, phosphate pH 7.3, 0.044 *M*, glycogen 0.4 mg/ml, nicotinamide 0.028 *M*, α-ketoglutaric acid, 0.0025 *M* and $MgCl_2$ 0.007 *M*. More recent experiments indicate that sucrose 0.25 *M* may be substituted for the above-described medium. The brain homogenization medium consists of sucrose 0.25 *M*, nicotinamide 0.025 *M*, glucose 0.001 *M*, α-ketoglutaric acid 0.0025 *M* and cytidine and uridine monophosphates 0.0002 *M*, adjusted to pH 7.3.

The liver mince was homogenized approx. 30 sec in a volume of medium twice its weight and brain mince in a volume 10 times its weight by four passes with the loose and tight fitting pestles. The liver homogenate was centrifuged in the number 30 rotor of the Spinco preparative ultracentrifuge at 800 × *g* for 10 min and the sedimented material discarded. This step was repeated and the supernatant was then centrifuged at 8500 × *g* for 10 min. The supernatant was discarded and the sedimented fraction was gently suspended in several milliliters of homogenizing medium. The suspension was mixed thoroughly with additional homogenizing medium to give a final volume equal to that from which the particulate material had been sedimented. The suspension was again centrifuged at 8500 × *g* for 10 min and the washing procedure repeated a second time. The twice-washed particles were gently suspended in homogenizing medium and promptly used.

The brain homogenate was centrifuged for 15 min at 800 × *g*, the sediment discarded and the supernatant re-centrifuged at 1500 × *g* for 15 min. The residue was rejected and the supernatant was then centrifuged at 12,000 × *g* for 15 min. The pale, lipid-like material surrounding the dark core was dislodged with a stirring rod and discarded with the supernatant. The dark residue was washed once with one-half the volume of the previous suspension and the pale sediment and supernatant were again discarded. The remaining particulate material was taken up in homogenizing medium for immediate use.

Preparation of substrate

A 150-g rat was fasted 2 days and then injected intraperitoneally with 0.1 mC each of DL-[2-^{14}C]lysine HCl and DL-[3-^{14}C]phenylalanine. (In some experiments uniformly labeled L-[^{14}C]phenylalanine was used.) 10 h later the animal was exsanguinated and the crude serum albumin fraction was obtained by 50 % saturation with neutral ammonium sulfate. After removal of the globulins, the solution was made 0.01 M in unlabeled phenylalanine and lysine, 0.01 M in EDTA and 0.001 M in glutathione. It was then dialyzed against distilled water, lyophilized, dissolved in distilled water and placed on a diethylaminoethyl cellulose column previously standardized with crystalline bovine serum albumin. The rat albumin was eluted with a soln. of 0.05 M NaCl and 0.005 M NaH_2PO4 at pH 6.5. A major peak of albumin was obtained, followed by appreciable tailing. The latter material was discarded. Elution of albumin was determined primarily by the highly specific vasoflavin fluorescence enhancement method[13, 14] and further checked by the radioactivity and 280 mμ absorption of the eluted fractions. The protein solution was lyophilized, dialyzed against distilled water and stored in the frozen state. Paper electrophoresis of this solution showed no detectable component except a single band corresponding to serum albumin. Addition of an aliquot to normal rat serum followed by paper electrophoresis yielded negligible radioactivity in all bands except that corresponding to serum albumin. Labeled albumin concentration was determined spectrophotometrically at 280 mμ by reference to crystalline bovine serum albumin as standard.

Incubation procedure

All incubations were conducted for a standard period of 2 h in a Dubnoff shaker at 37° in air. Incubation was begun as soon as possible after the particulate fractions were obtained. These were added in 1-ml aliquots to pre-loaded beakers containing labeled albumin and other factors as specified in the protocols.

Preparation of samples for counting

Perchloric acid (PCA)-soluble fractions: One-half of the contents of each incubation beaker was removed at zero time and precipitated with 6% PCA. After incubation the remaining portions were similarly treated. The precipitated protein was allowed to stand overnight in the cold and then removed by centrifugation. The residues were washed once with 6% PCA and the washes were combined with the respective supernatants. 10 mg of unlabeled albumin was added to each sample, carefully triturated into fine suspension and allowed to stand in the cold for a minimum of 6 h. The samples were centrifuged and the supernatant solutions were decanted and neutralized with 6 N KOH. The $KClO_4$ was removed by centrifugation and washed with a small vol. of cold water. The combined washes and supernatants were evaporated to dryness in stainless steel planchets. For accurate comparison, the activity of the protein used in the experiment was determined by incubating a beaker with all components except labeled protein and processing this blank with the other samples. An aliquot of the original labeled albumin was added to the final neutralized supernatant from this blank prior to evaporation and then counted.

Protein samples: Precipitates remaining after the 6% PCA wash were suspended in 0.02 M acetate buffer pH 4.7 in 50% methanol and centrifuged. The residues were then washed with acetone, heated 15 min at 50° in alcohol–ether (3:1, v/v), centrifuged and then washed with ether. The protein was centrifuged and the ether was discarded. The residues were suspended in a solution of chloroform–methanol–conc. HCl (200:100:1, v/v). The suspension was poured into a pre-weighed planchet and gently evaporated to dryness. Protein counts were corrected to infinite thinness by determining the sample weight and reference to an unpublished calibration curve. All protein dry wt. determinations were performed in a similar manner.

Determination of radioactivity was performed in a Nuclear automatic gas flow counter. All samples were counted to within 5 % error.

All solutions added to the incubation systems were adjusted to neutral pH before use. GSH, cysteine and thioglycollate solutions were prepared just prior to incubation.

Materials

ATP, UTP, CTP, GTP, AMP, DPN, DPNH, TPN and CoA were obtained from Sigma Laboratories. GSH, cysteine and α-ketoglutaric acids were products of the California Foundation for Biochemical Research. Albumin (crystalline and Fraction V) was obtained from Mann Research Laboratories. Diethylaminoethylcellulose was purchased from Eastman Kodak Company. DL-[3-^{14}C]Phenylalanine and DL-[2-^{14}C]-lysine were obtained from Tracerlab. Uniformly labeled L-[^{14}C]phenylalanine was furnished by Dr. L. L. MILLER through the agency of the Rochester Atomic Energy Commission.

Chromatography

Two chromatographic systems were employed: 95 % ethanol–glacial acetic acid (100:1, v/v), and the upper layer from butanol–acetic acid–water (4:1:5,v/v). In all instances unlabeled phenylalanine was co-chromatogrammed with a small aliquot of the solution under investigation. Use of this strip as guide permitted localization of the phenylalanine area despite variations in R_F due to other components such as salts. All chromatographic procedures were carried out at 5°.

RESULTS

Examination by phase microscopy of the preparations from liver and brain used in these experiments reveals mitochondria and essentially no contamination by whole

TABLE I

ATP REQUIREMENT IN SERUM ALBUMIN CATABOLISM*

System	Liver**			Brain***		
	Zero time	*Incubation products*	Δ	*Zero time*	*Incubation products*	Δ
Complete	13	187	174	32	124	92
— ATP	11	93	82	28	53	25
— ATP + GTP	10	78	68	24	43	19
— ATP + UTP	15	95	80	35	62	27
— ATP + CTP	12	89	77	32	65	33
— ATP + AMP	14	80	66	30	59	29
— ATP + DPN	16	147	131	27	96	69
— ATP + DPNH	12	155	143	35	98	73
— ATP + TPN	15	118	103	26	82	56
— ATP — CoA	14	22	8	31	35	4

* PCA-soluble, counts/min.

** The complete system contained 1.0-ml particulate fraction, 11.4 mg dry wt., 4 mg CoA, 13 μmoles GSH, 4 μmoles ATP, 5.2 mg rat albumin counting 2300 counts/min, in a total vol. of 3.0 ml. 4 μmoles of each nucleotide substitution for ATP were present.

*** The complete system contained 1.0 ml particulate fraction, 27 mg dry wt., and 1.6 mg rat albumin counting 5980 counts/min, in a total vol. of 3.0 ml. CoA, GSH, ATP and other additions as in liver system.

cells, cell fragments or nuclei. It was found that this particulate fraction from either liver or brain gave no significant increase in PCA-soluble activity when incubated with labeled rat-serum albumin. Addition of ATP and CoA caused an appreciable increase in the PCA-soluble activity on incubation. Concentrations of DPN, DPNH and TPN equivalent to that of ATP also stimulate production of PCA-soluble activity, though less markedly. GTP, CTP, UTP and AMP do not substitute for ATP. It would appear that the liver fraction is considerably more active than that derived from brain, on the basis of defatted dry wt. comparisons of the respective fractions. These data are summarized in Table I. In both the liver and brain systems, the catabolism of serum albumin is strongly inhibited by cyanide (10^{-3} *M*) or azide (10^{-2} *M*), suggesting that degradation of the protein is dependent on cellular respiration. Addition of dinitrophenol (10^{-3} *M*) and arsenite (10^{-3} *M*) to the incubation system has a similar inhibitory effect, as shown in Table II.

The increase in the PCA-soluble counts on addition of CoA is highly variable but can be stabilized by use of glutathione. When cysteine was used at levels equivalent to glutathione, with or without CoA, the system was inhibited. Neither glutathione, thioglycollate nor cysteine can substitute for CoA. These data are summarized in Table III.

TABLE II

DEPENDENCE OF SERUM ALBUMIN CATABOLISM ON RESPIRATION*

System	Liver**			Brain***		
	Zero time	Incubation products	Δ	Zero time	Incubation products	Δ
Complete	21	99	78	31	67	36
+ CN	23	29	6	35	39	4
+ Azide	19	30	11	34	39	5
+ DPN + Arsenite	24	49	25	37	46	9

* PCA-soluble, counts/min.

** Complete system as in protocol for Table I, except for rat albumin 1.2 mg, 640 counts/min; 8.8 mg dry wt. particulate fraction.

*** Complete system as in protocol for Table I, except for rat albumin, 3.6 mg, 1920 counts/min; 19 mg dry wt. particulate fraction.

TABLE III

CoA DEPENDENCE OF SERUM ALBUMIN CATABOLISM*

System	Liver**			Brain***		
	Zero time	Incubation products	Δ	Zero time	Incubation products	Δ
Complete	26	118	92	29	55	26
— GSH	24	75	51	30	46	16
— CoA	28	52	24	33	41	8
— GSH + Thioglycollate	26	62	36	28	34	6
— GSH + Cysteine	26	33	7	33	34	1
— GSH — CoA + Cysteine	23	28	5	30	32	2

* PCA-soluble, counts/min.

** Complete system as in protocol for Table I, except for rat albumin, 1.2 mg, 640 counts/min; 6.7 mg dry wt. particulate fraction. 13 μM thioglycollate and cysteine substituted.

*** Complete system as in protocol for Table I, except for rat albumin, 2.4 mg, 1280 counts/min; 14.7 mg dry wt. particulate fraction.

The microsomal fraction obtained by centrifugation of the liver 8000 × g supernatant for 30 min at 100,000 × g does not metabolize added serum albumin. When the microsomal preparation is added to the active fraction from liver, formation of PCA-soluble products from albumin is powerfully inhibited. The inhibition is not due to transfer of radioactivity to the microsomal fraction. Table IV shows only minor increases in the activity of the added microsomes isolated from the sample mixtures at zero time and after incubation. In addition, these increases are unaffected by the presence of the cofactors required for albumin metabolism. The control system shows the extent of PCA-soluble product formation in the absence of added microsomes. The increase in microsomal count, about 17 counts/min, does not account for the drop in the PCA-soluble counts from 172 to about 26.

TABLE IV

MICROSOMAL INHIBITION OF ALBUMIN CATABOLISM

System*	PCA-soluble counts/min			Microsome protein counts/min		
	Zero time	*Incubation products*	Δ	*Zero time*	*Incubated protein*	Δ
Complete	38	210	172			
— ATP — CoA	34	72	38			
Complete + Microsomes	32	55	23	33	47	14
— ATP — CoA	36	64	28	29	46	17
Complete — P.F. + Microsomes	47	52	5	28	43	15
— ATP — CoA	38	45	7	32	41	9
	Inhibition: 172 — 26 = 146			Incubation: 14		

* Liver fractions only were used. Protocol as in Table I, except for rat albumin, 4.2 mg, 1350 counts/min; 8.9 mg dry wt. particulate fraction (P.F.), 9.4 mg microsomal dry wt.

When uniformly labeled phenylalanine is used as the isotopic source, and the perchloric acid soluble products from liver and brain are chromatogrammed on paper, approx. 60–70% of the total radioactivity (Table V) moves with the same R_F as phenylalanine in two solvent systems. Elution of the phenylalanine region obtained by use of the ethyl alcohol–acetic acid system, followed by lyophilization and chromatography with butanol–acetic acid, yielded the major percentage of the total activity in the area corresponding to phenylalanine. In the ethyl alcohol–acetic acid,

TABLE V

DISTRIBUTION OF ACTIVITY AMONG INCUBATION PRODUCTS*

	Liver	*Brain*
Decrease in Protein	271	—
Increase in PCA-Soluble	258	63
Free Phenylalanine (R_F 0.34)	152	42
Bound Phenylalanine (R_F 0.17)	64	9
Activity at Origin	22	5

* All values at infinite thinness, counts/min.

the remainder of the activity is distributed in two other bands which quench strongly under u.v. The first of these components remains at the origin and the second moves with an R_F of 0.17 (R_F of phenylalanine is 0.34).

The R_F 0.17 component(s) is ninhydrin negative when sprayed with ninhydrin–acetone. On elution, it yields about 25 % of the total PCA-soluble counts. At pH 7 the spectrum shows a single peak in the u.v. region at 258 mμ and a 280/260 ratio of 0.23. On hydrolysis overnight in 6 N HCl this material yields a labeled product which moves with the R_F of phenylalanine in the two solvent systems. Other ninhydrin-positive components also appear after hydrolysis. Measurement of the protein activity at zero time and after incubation shows reasonable correspondence between the decrease in protein counts and the rise in PCA-soluble radioactivity. These data are summarized in Table V. The further resolution and characterization of the components present in these 2 bands will be the subject of future communication.

It will be noted that in all protocols the volume of particulate suspension added to the incubation sample is 1.0 ml and that the final vol. is 3.0 ml. 1 ml H_2O must be included. Only minor variations in this ratio are permissable without appreciable inhibition. Under these conditions, the optimal levels of CoA, GSH and ATP are about 4, 4 and 3 mg respectively for 10 mg dry wt. of the particulate fraction from liver and 20 mg of that from brain.

DISCUSSION

The report by SIMPSON[15] of the catabolism of endogenous protein in liver slices at neutral pH showed that the liberation of free isotopic amino acid was dependent on oxidative processes. Extension of these observations by STEINBERG AND VAUGHAN[16] suggested that more than one degradative pathway existed in liver slices. At pH 7, catabolism of endogenous protein was inhibited by anaerobiosis and dinitrophenol, whereas at pH 5 dinitrophenol had no effect. ANSELL AND RICHTER[17] reported on a neutral proteinase in rat brain mince whose activity apparently was not dependent on respiration but which was detectable for only a short period after sacrifice of the animal. The unresponsiveness of this proteinase to cysteine and ferrous ion suggests that it differs from the enzyme in mouse-brain particles observed by KRIMSKY AND RACKER which was capable of degrading endogenous phosphoglyceraldehyde dehydrogenase[18].

It appears that several mechanisms for protein breakdown exist in tissue and that, at physiological pH, one group of these is ultimately dependent on the respiratory mechanism. A plausible consequence of this requirement would be the formation of activated intermediates which might also be readily re-utilized. The experiments of LOFTFIELD AND HARRIS have yielded data in accord with this possibility[19]. In the intact rat, amino acids from tissue (or serum) protein were incorporated more efficiently into newly formed ferritin than was administered amino acid. The breakdown of pre-existent protein would thus appear to be an important factor in the formation of new protein by the mammal.

In this study, serum albumin was selected as a model protein for the investigation of the catabolic process. The information obtained must be qualified by the appreciation that the metabolism of the extracellular serum albumin may not be analogous to that of the intracellular protein.

Certain qualitative similarities however, between the albumin degradations reported here and the catabolism of endogenous protein in tissue slices[15,16] should be noted. These are (a) dependence on the respiratory mechanism in both cases; (b) formation of free amino acid; (c) inhibition by agents which uncouple oxidative phosphorylation; (d) occurrence at a physiological pH.

The cofactor requirements for breakdown indicate that some stage in the reaction sequence differs from hydrolysis. ATP and CoA may be required for synthesis of an unknown cofactor(s) which is directly involved in the process, or for the binding of the protein to the particle, or for actual scission of the peptide bond. The data do not permit selection among these possibilities. The occurrence of products other than free phenylalanine, associated with characteristic nucleotide spectra, encourage the consideration of the last alternative. It is apparent, however, that there is both a physical and chemical segregation of the catabolic pathways available for different types of protein molecules. Insulin, α-corticotropin and ribonuclease can be degraded by homogenate fractions in the absence of added cofactors. These fractions exhibit no catabolic activity toward serum albumin[10,11].

Three lines of evidence suggest that the metabolic systems described here may be of general significance. The bound phenylalanine revealed by chromatography may bear close relation to the nucleotide-peptide compounds isolated from rat liver[20,21], yeast[22,23] and bacteria[24,25]. It is possible that the general occurrence of such compounds reflects a pattern of reactions in the intermediary metabolism of the protein molecule common to these widely divergent sources.

The liver has been shown to be the site of albumin synthesis[26] and to be involved in its catabolism[27]. It is of interest that similar cofactor requirements are found for the *in vitro* catabolic systems derived from both liver and brain. Similar enzymic systems are thus implied in two functionally differing mammalian tissues.

Finally, the rate at which serum albumin is catabolized by the liver fraction *in vitro* is sufficient to account for the major proportion of the albumin turnover in the intact animal. Taking the half-life of serum albumin in a 200-g rat as 3.5 days[28,29] and the amount as 150 mg, then approx. 0.9 mg is broken down per hour From Table I it can be calculated that 0.39 mg of albumin is converted to PCA-soluble products in 2 h. The incubated particulate material was derived from 1.7 g liver. Assuming the weight of liver in a 200-g rat to be 6 g, then the whole liver can degrade $6/1.7 \cdot 0.39 \cdot 1/2$ mg/h or about 0.7 mg albumin/h. The activity of the *in vitro* system thus suggests that it is a potential major pathway for albumin catabolism in the intact animal.

The site of catabolism appears to be the mitochondria. The possibility cannot be excluded that some specific subfraction of the microsomes, isolated with the mitochondria, may exercise a synergistic or major role. Some special characteristic of this subfraction would be required since the microsomes obtained by centrifugation at 100,000 × *g* for 30 min do not metabolize added serum albumin and inhibit catabolism on their addition to the active system.

It is not possible at present to explain the disparity in activity between the liver and brain fractions. The nature and amount of enzymes present, requirements for other unknown factors, and difficulty in preparative procedures may all contribute to lowered activity in the isolated brain system.

The appearance of free phenylalanine as the major component in the PCA-soluble products from both liver and brain (Table V) indicates that it is actually protein which is catabolized rather than adventitious contaminants. Treatment of guide strips with ninhydrin reveals a number of reactive components in addition to phenylalanine, but these have not yet been identified.

The inhibitory effect of cysteine observed in this study does not appear to be unique. This effect has been described for another coenzyme A-requiring system involved in protein metabolism[30]. The mechanism by which DPN and TPN partially substitute for ATP is under investigation.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. A. White for his support and encouragement in the prosecution of this study, to Dr. J. Betheil, Department of Biochemistry, Albert Einstein College of Medicine, for the gift of vasoflavin, and to Dr. A. Novikoff of the department of Pathology, Albert Einstein College of Medicine, for the examination of samples by phase microscopy.

This work was performed while the author was a senior post-doctoral fellow on Interdisciplinary Grant 2M-6418, National Institute of Mental Health, Public Health Service.

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STUDIES ON CYTOCHROME *a*

IV. REACTION OF CYTOCHROME *a* WITH CYTOCHROMES *c* AND c_1

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(Received February 13th, 1959)

SUMMARY

In the present paper, the important role played by cytochrome *c* in the cytochrome oxidase system is demonstrated by using highly purified preparations of cytochromes *a*, *c*, and c_1. Despite the fact that cytochrome *a* can be rapidly reduced by *p*-phenylenediamine, the component is not oxidized by oxygen if cytochrome *c* is not present. The K_m calculated for cytochrome *c* is similar to that found in particulate preparations. The addition of cytochrome c_1 has no effect on the cytochrome oxidase reaction. The reaction between cytochromes c_1 and *a* is very slow and is mediated by cytochrome *c*. On the basis of the above results, it is proposed that cytochrome *c* acts not only as an electron carrier between cytochromes c_1 and *a* but also as an essential constituent to form an active complex with cytochrome *a* in the cytochrome oxidase system. The reaction mechanism of the cytochrome oxidase system is discussed on the basis of the above results.

INTRODUCTION

While studying the spectral properties of highly purified cytochrome *a*, it was found[1] that the dithionite-reduced form of cytochrome *a* can combine with oxygen as evidenced by shifts of the γ-band from 444 mμ to 426–28 mμ and the α-band from 605 mμ to 603 mμ with accompanying changes in absorbancy. However, it could not be oxidized unless cytochrome *c* was present in the reaction system. In the presence of cytochrome *c*, the preparation showed a strong cytochrome oxidase activity and this activity, as well as that of native cytochrome oxidase, was inhibited by cyanide and carbon monoxide[2]. On the basis of the above results, it seems likely that cytochrome *a*, *i.e.*, our cytochrome oxidase preparation, is not autoxidizable unless a small amount of cytochrome *c* is present in the preparation. These findings led us to the conclusion that cytochromes *a* and *c* cooperate to produce cytochrome oxidase activity not only in the electron transfer but also in the formation of an active complex having strong autoxidizability[3,4].

With regard to the reactions of cytochrome *a* with cytochrome *c* or c_1, YAKUSHIJI AND OKUNUKI[5,6] found by using partially purified components that (a) electron transfer between cytochromes *a* and *c* is reversible and the rate of transfer from *c* to *a* is very rapid and (b) the rate of transfer from c_1 to *a* is very slow. Their findings and the important role of cytochrome c_1 in the typical electron-transferring system were later confirmed by the investigations of BALL AND COOPER[7], WIDMER *et al.*[8], KEILIN AND HARTREE[9], and ESTABROOK[10]. Particularly the last investigation, in which the quantitative relations between cytochromes *a*, *c* and c_1 in liver non-phosphorylating particles were assayed by means of a sensitive spectrophotometer, agreed well with those of YAKUSHIJI AND OKUNUKI[5,6].

The present paper deals with the reaction of cytochrome *a* with cytochromes *c* and c_1 from the standpoint of the cytochrome oxidase activity. Quantitative results are given for the relation between these three components, purified by our method from beef heart. Furthermore, the reaction mechanism of the cytochrome oxidase system is discussed on the basis of these results.

PREPARATIONS AND MATERIALS

Cytochrome a

Cytochrome *a* was purified by means of cholic acid and fractionation with ammonium sulfate according to the method described previously[11]; the nomenclature of the fractions is the same as before[3,11].

Cytochrome c

Crystalline cytochrome *c* in the oxidized state was prepared from beef-heart muscle according to the method of HAGIHARA *et al.*[12] and recrystallized once. The preparation was dissolved in 0.1 *M* phosphate buffer, pH 7.2, dialyzed against the same buffer overnight at 5°, and stored in the frozen state at $-20°$.

Cytochrome c_1

Cytochrome c_1 was purified according to a modification of our previous method[13]. The outline of this method is as follows; To 400 ml of particulate preparation was added 100 ml 10 % cholic acid, dissolved in 0.1 *M* phosphate buffer and adjusted to pH 7.2, with 4 *N* NaOH, and 125 ml of saturated ammonium sulfate buffered to pH 7.2 with conc. NH_4OH. The mixture was stored in a cold room at 5° for 24–36 h and then centrifuged at $8,000 \times g$ for 25 min. The cloudy supernatant fluid was decanted and the precipitate was discarded. To the supernatant fluid saturated ammonium sulfate solution was added to 50 % saturation. The resulting precipitate was collected by centrifugation at $9,000 \times g$ for 25 min and dissolved in 100 ml 0.1 *M* phosphate buffer containing 2 % cholic acid, pH 7.2. Saturated ammonium sulfate was added to 15 % saturation. The mixture was allowed to stand at 5° for 72–96 h and then centrifuged. The temperature of the slightly turbid supernatant fluid, which contained cytochromes *a*, *b* and c_1, was raised to 40° in a water bath and was maintained at this temperature for 15–20 min. The solution was chilled and recentrifuged. By this treatment, cytochrome *b* was denatured and removed from the preparation. To the clear supernatant fluid, saturated ammonium sulfate was added to raise the concentration to 48 % saturation. The precipitate was collected by centrifugation, dissolved in a small amount of 0.1 *M* phosphate buffer containing 1 % cholic acid, pH 7.2, and dialyzed against 0.01 *M* phosphate buffer containing 0.2 % cholic acid, pH 7.2, at 5° for 5 h. After the dialysis, cytochrome *a* was precipitated and removed by centrifugation. The solution of cytochrome c_1 was immediately treated with alumina C_γ gel and eluted from the gel with 20 % saturated ammonium sulfate containing 1 % cholic acid at pH 7.2. Cytochrome c_1 was further concentrated by fractionation with ammonium sulfate and dialyzed against 0.1 M phosphate buffer containing 0.5 % cholic acid, pH 7.2, at 5° for 4 h and stored in an ice box at 4°. The yield of cytochrome c_1 is about 2–3 times greater in this method than in the former method and contamination by flavine is almost negligible.

Reduced cytochromes c *and* c₁

Cytochrome c or c_1 obtained by the above methods was reduced by addition of a small amount of $Na_2S_2O_4$ and the excess of dithionite was removed from the preparation by vigorous aeration for 10–15 min. The preparation could also be reduced by the Pd-H_2 method.

Cytochrome c*-free succinic oxidase system*

As the cytochrome c-free succinic oxidase system, the ether-treated particulate preparation and fraction S_2 of cytochrome a were used. The ether-treated particulate preparation was prepared from an ordinary heart-muscle preparation by washing twice with ether and then thrice with 0.1 M phosphate buffer, pH 7.4. In this way, cytochrome c was completely removed from the preparation, but the x-factor[6] was only partially removed, and fairly high activity was obtained on addition of cytochrome c. The fraction S_2 was used after washing well with 55 % saturated ammonium sulfate solution at 5°.

Other materials used were the same as described in the preceding paper[2].

METHODS

Manometric assay

Experimental conditions for the manometric assay for cytochrome oxidase activity were the same as reported before[2]. For succinic oxidase, the conditions used were the same as above except that 0.2 ml 0.1 M sodium succinate was used as substrate.

Spectrophotometric assay

Spectrophotometric measurements were performed in a Shimadzu spectrophotometer type QB-50, Hitachi automatic recording spectrophotometer type EPS-2, or Cary spectrophotometer Model 14, using a cuvette of 1-cm light path. The experimental conditions are described in each section.

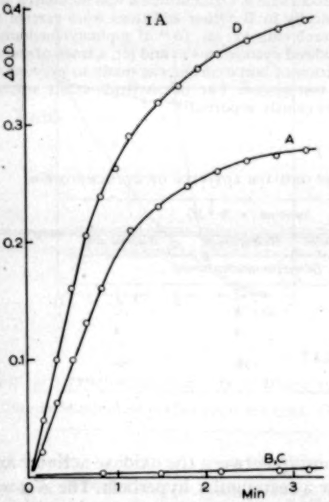
Determination of the concentration of cytochromes

The concentration of cytochromes used in these experiments were determined spectrophotometrically based on the following extinction coefficients[2, 11, 13]; cytochrome a, $\Delta\varepsilon_{605\,\mathrm{m}\mu-630\,\mathrm{m}\mu\,(\mathrm{reduced})} = 19.5\ \mathrm{m}M^{-1}\cdot\mathrm{cm}^{-1}$; cytochrome c, $\varepsilon_{550\,\mathrm{m}\mu\,(\mathrm{reduced})} = 27.7\mathrm{m}M^{-1}\cdot\mathrm{cm}^{-1}$; cytochrome c_1, $\varepsilon_{553\,\mathrm{m}\mu\,(\mathrm{reduced})} = 15.8\ \mathrm{m}M^{-1}\cdot\mathrm{cm}^{-1}$.

RESULTS

Autoxidizability of cytochrome a

In Fig. 1 A is shown the time course of the reduction of cytochrome a with 0.001 M p-phenylenediamine, hydroquinone, or ascorbic acid, respectively. Under these experimental conditions, *p*-phenylenediamine can reduce cytochrome *a* about 50 times more rapidly than the other two reductants*. As shown in Fig. 1 B, in which the time course of the reduction of the α-band of cytochrome *a* with various concentrations of *p*-phenylenediamine is measured, 0.01 *M* *p*-phenylenediamine can completely reduce the α-band within 1 min. From the above results, *p*-phenylenediamine is judged to be a good reductant of cytochrome *a*, as reported by SMITH[14]. Despite this fact, *p*-phenylenediamine-reduced cytochrome *a* is not oxidized by oxygen if a small amount of cytochrome *c* is not present in the reaction system as shown in Fig. 2. These facts were also confirmed manometrically. As illustrated in Table I, oxygen is not taken up by cytochrome *a* in the presence of the above reductants. By the addition of a sufficient amount of cytochrome *c*, however, cytochrome *a* can consume a large amount of oxygen with the above reductants. Based on the above results, it appears that cytochrome *a* does not show any appreciable autoxidizability without cytochrome *c*.



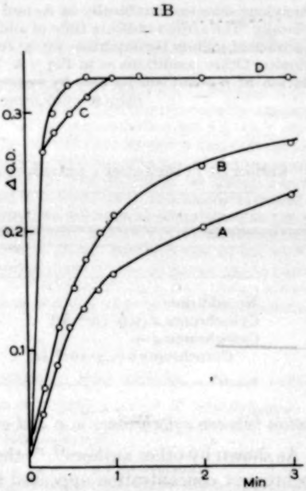


Fig. 1 A. Reduction of cytochrome *a* by *p*-phenylenediamine, hydroquinone, or ascorbic acid. The sample cuvette contained 2.7 ml cytochrome *a* solution diluted with 0.1 *M* phosphate buffer containing 1 % Emasol 4130, pH 7.4 ($4.6 \cdot 10^{-6}$ *M* cytochrome *a*). The reaction was initiated by the addition of 0.3 ml of 0.01 *M* solution of each reductant. The reduction was determined by measuring the increase in absorbancy of the α-band at 605 mμ or of the γ-band at 444 mμ in a Cary spectrophotometer at 30°. Curves A, B, and C show the reduction of the α-band by *p*-phenylenediamine, hydroquinone, and ascorbic acid, respectively. Curve D shows the reduction of the γ-band by *p*-phenylenediamine, at a concentration of cytochrome *a* one third of that in the other curves.

Fig. 1 B. Reduction of cytochrome *a* by various concentrations of *p*-phenylenediamine. Experimental conditions as in Fig. 1 A, except that the measurements were made in a Hitachi spectrophotometer at 15°. Curve A, reduction of the α-band by 10^{-3} *M* *p*-phenylenediamine; Curve B, $2 \cdot 10^{-3}$ *M* *p*-phenylenediamine; Curve C, 10^{-2} *M* *p*-phenylenediamine and Curve D, by 10^{-2} *M* *p*-phenylenediamine plus 10^{-2} *M* ascorbic acid, respectively.

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* The result presented in the preliminary report[4] was uncertain due to colour developed in the blank.

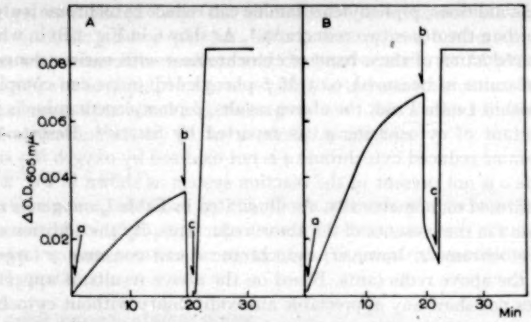


Fig. 2. Effect of cytochrome *c* on the oxidation of cytochrome *a*. Cytochrome *a* was incubated with *p*-phenylene diamine aerobically in A, and anaerobically in B. Other additions were carried out aerobically. The arrows indicate time of addition of each reagent; (a), 10^{-4} *M* *p*-phenylenediamine and a trace of sodium borohydride; (b), $5 \cdot 10^{-9}$ *M* oxidized cytochrome *c*; and (c), a trace of sodium dithionite. Other conditions as in Fig. 1 A. The addition of borohydride was made to prevent the oxidation of *p*-phenylenediamine by oxygen in the test system, but borohydride itself scarcely reduces cytochrome *a* as previously reported[11].

TABLE I

EFFECT OF CYTOCHROME *c* ON THE CYTOCHROME OXIDASE ACTIVITY OF CYTOCHROME *a*

	Substrate ($\times 10^{-3}$ *M*)		
System	*p-Phenylenediamine*	*Hydroquinone*	*Ascorbic acid*
	(*μl oxygen uptake/10 min*)		
No addition	4	4	10
Cytochrome *a* ($0.9 \cdot 10^{-5}$ *M*)	6	4	4
Cytochrome *a* + Cytochrome *c* ($1.4 \cdot 10^{-5}$ *M*)	104	126	80

Relation between cytochromes a, c *and* c_1

As shown by other authors[15,16], the relationship between the oxidase activity and cytochrome *c* concentration appeared to follow a rectangular hyperbola. The K_m was not altered by the addition of cytochrome c_1. Fig. 4 shows the time course of oxidation of reduced cytochrome *c* or c_1 by cytochrome *a*. Reduced cytochrome *c* is rapidly oxidized by cytochrome *a*, while reduced cytochrome c_1 is hardly oxidized. On the addition of a small amount of cytochrome *c* to the latter reaction system, however, cytochrome c_1 is also oxidized rapidly. The results indicate that cytochrome *a*, in the absence of cytochrome *c*, can oxidize reduced cytochrome c_1 at about 1/30–1/50 the rate of reduced cytochrome *c*.

The fact that cytochrome c_1 cannot act as a substrate for cytochrome *a* and that cytochrome *c* is necessary to link electron transfer between cytochromes *a* and c_1 were further confirmed manometrically using the cytochrome *c*-free succinic oxidase system. An active preparation prepared by ether treatment or fraction S_2 shows no oxygen uptake if cytochrome *c* is not added, as illustrated in Table II. These facts are observed when either succinate or *p*-phenylenediamine is used as substrate. The results also strongly supported the conclusion that cytochrome *c* is the only component which can act as an electron donor for cytochrome *a* in the cytochrome oxidase system.

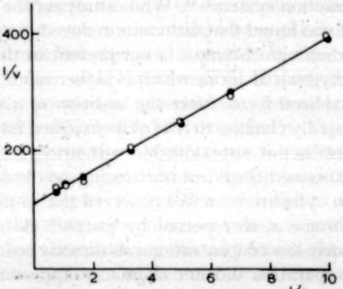


Fig. 3. Effect of cytochrome c_1 on cytochrome oxidase activity of cytochromes *a* and *c*. The reaction system contained $0.5 \cdot 10^{-5}$ *M* cytochromes *a* and c_1 in 1 % Emasol 4130. The activity was measured manometrically using hydroquinone as substrate at 30°. Abscissa, reciprocal of molarity of cytochrome *c* ($\times 10^5$); ordinate, reciprocal of velocity of reaction ($\times 10^{-4}$); ○, + cytochrome c_1; ●, — cytochrome c_1.

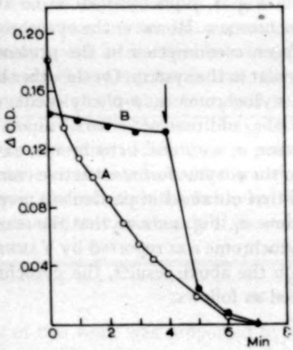


Fig. 4. Oxidation of reduced cytochrome *c* or c_1 by cytochrome *a*. The sample cuvette contained $3.3 \cdot 10^{-2}$ *M* phosphate buffer, pH 7.4, 0.5 % cholic acid, $1.4 \cdot 10^{-6}$ *M* cytochrome *a* and cytochromes *c* and c_1. The total vol. was 3.0 ml and the temperature was 15°. The oxidation was initiated by the addition of cytochrome *a* and was determined by measuring the decrease in absorbancy at 550 mμ for cytochrome *c* and at 553 mμ for cytochrome c_1. Curve A shows the oxidation of $0.9 \cdot 10^{-5}$ *M* cytochrome *c* and Curve B shows the oxidation of $1.4 \cdot 10^{-5}$ *M* cytochrome c_1. The arrow shows the time of addition of $2 \cdot 10^{-6}$ *M* cytochrome *c*.

TABLE II

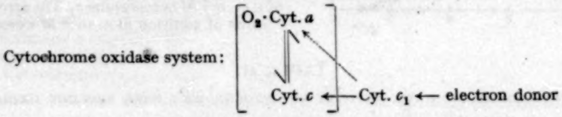
EFFECT OF CYTOCHROME *c* ON THE ACTIVITY OF CYTOCHROME *c* FREE SUCCINIC OXIDASE SYSTEM

For the method of preparation see text. The system contained $0.7 \cdot 10^{-5}$ *M* cytochrome *c*.

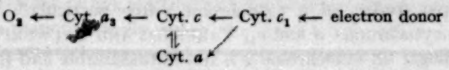
Preparation	*Substrate (10^{-2} M)*			
	Succinate		*p-Phenylenediamine*	
	— Cyt. c	*+ Cyt. c*	*— Cyt. c*	*+ Cyt. c*
	(μl oxygen uptake/30 min)			
Ether-treated particulate preparation	5	115	2	137
Fraction S_2	0	61	0	73

DISCUSSION

From spectroscopic studies of a cytochrome *c*-free particulate preparation and partially purified cytochromes *a* and c_1, YAKUSHIJI AND OKUNUKI[5,6] have reported the following findings; (a) cytochrome *a* is not autoxidizable and (b) because of the slow rate of transfer from c_1 to *a*, electron transfer from cytochrome c_1 to cytochrome *a* is mediated by cytochrome *c*. Recently, we have succeeded in preparing a highly purified cytochrome *a* preparation having a strong cytochrome oxidase activity, by an improvement of the method of YAKUSHIJI AND OKUNUKI[17] and by removal of inhibition of cholate by the addition of Emasol 4130 (a non-ionic detergent) to the reaction system[2, 11]. While studying the spectral properties of the above preparation, it was found that dithionite-reduced cytochrome *a* is not oxidized by oxygen. However, when cytochrome *c* is not present in the system, it shows a spectrum similar to the oxygenated form, which is rather more like that of the CO-complex than that of the oxidized form. After the addition of a small amount of cytochrome *c*, the spectrum rapidly changes to that of its oxidized form[1, 3]. These facts suggest that our preparation also is not autoxidizable without cytochrome *c*. To confirm these facts further, we examined the effect of reducing agents other than dithionite or reduced cytochrome *c* on cytochrome *a*. We observed that *p*-phenylenediamine is a good reductant of cytochrome *a*, as reported by SMITH[14]. As shown in Fig. 1, *p*-phenylenediamine at the fairly low concentration can directly reduce cytochrome *a*. However the cytochrome *a* preparation did not show any appreciable oxygen consumption in the presence of *p*-phenylenediamine if cytochrome *c* was not present in the system. On the other hand, as reported in the case of dithionite-reduced cytochrome *a*, *p*-phenylenediamine-reduced cytochrome *a* is rapidly oxidized on the addition of a small amount of cytochrome *c*. Thus, it may be that cytochrome *a*, *i.e.*, our cytochrome oxidase preparation, is strongly autoxidizable only when the enzyme forms an active complex with cytochrome *c*. The K_m is closely similar to that obtained in particulate preparations and not altered by the addition of cytochrome c_1. Fig. 4 shows that the reaction between cytochromes *a* and c_1 is mediated by cytochrome *c* as reported by YAKUSHIJI AND OKUNUKI[5, 6] and ESTABROOK[10]. Based upon the above results, the cytochrome oxidase system may be schematically represented as follows;



Since KEILIN AND HARTREE[18] proposed the entity, cytochrome a_3, it has been considered that cytochrome a_3 is autoxidizable and is identical with cytochrome oxidase. This consideration was strongly supported by the observation of CHANCE[19] that the rate of reduction or oxidation of the α-band at 605 mμ by substrate or oxygen does not exactly correspond to that of the γ-band at 444 mμ in the cytochrome oxidase preparation. If the above assumption is true, reduced cytochrome *a* should be rapidly oxidized by cytochrome a_3. However, to interpret the findings presented in Fig. 1 and 2 it would have to be concluded that cytochrome *c* mediates in the reaction between cytochromes *a* and a_3. On the other hand, it has been clearly demonstrated by many investigations that cytochrome *c* reacts as an intermediate between cytochromes c_1 and *a*. If the cytochrome oxidase preparation contains two cytochrome components, *i.e.*, *a* and a_3, these may be schematically represented as follows;



In this scheme, however, it may be more difficult to interpret the role of cytochrome *a* in a respiratory system.

Based upon the spectral properties and analyses of heme and other constitutions of our cytochrome *a* preparation[1,11], we have considered that our preparation contains only one heme component and that cytochrome *a* occupies a terminal position in the electron transferring system in a cooperation with cytochrome *c*. In our scheme shown above, the role of cytochrome *a* in the action of cytochrome oxidase is emphasized and cytochrome *c* acts not only as an electron donor but also as an essential constituent of cytochrome oxidase forming an active complex with cytochrome *a*. In fact, the O_2-cytochrome *a* complex is not observed in the presence of cytochrome *c*, but carbon monoxide competes with oxygen and thus competitively inhibits the activity. These facts may suggest that the mode of action of cytochrome *a* with oxygen is modified by the presence of cytochrome *c*. Unlike other non-autoxidizable cytochromes, the heme of cytochrome *a* functions as an electron acceptor for cytochrome *c* and as an activator of oxygen. From this standpoint therefore, cytochrome oxidase is not a simple electron carrier but an enzyme. We are now studying the physico-chemical properties and interaction between the protein moiety and heme of cytochrome *a*. If the above assumption is true, the properties of cytochrome oxidase now thought of as those of cytochrome a_3 will be better interpretable as those of cytochrome *a* and the difference between the cytochrome a_3 theory and the present theory will be largely a question of definition as pointed out by SLATER[20]. In other words, the problem will be concentrated on whether cytochrome a_3 is essentially different from cytochrome *a* or whether the behavior of the heme of cytochrome *a* is, in an extremely complicated way as a result of its reaction mechanism with cytochrome *c*, as discussed above.

ACKNOWLEDGEMENTS

Part of this work was supported by the grant of the Scientific Research Fund of the Ministry of Education.

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CREATINE PHOSPHORYL TRANSFERASE AND PHOSPHOGLYCERALDEHYDE DEHYDROGENASE IN IODOACETATE POISONED MUSCLE

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(Received March 10th, 1959)

SUMMARY

By extraction of iodoacetate poisoned muscles and subsequent fractionation and assay for enzymic activity, it has been shown that in such muscles phosphoglyceraldehyde dehydrogenase is completely inhibited, while creatine phosphoryltransferase is not affected at all, even though *in vitro* the latter enzyme is nearly as susceptible to such inhibitions as is the former.

This result is explained by the finding that several substances can protect creatine phosphoryltransferase against the action of iodoacetate. In decreasing order of effectiveness, these are: PC, ATP $>$ ADP, PP $>$ AMP $>$ P, while arsenate and creatine give no protection.

These findings eliminate the doubts that have recently been expressed as to the explanation of the phosphocreatine splitting in iodoacetate poisoned muscle, and thereby against the physiological significance of the Lohmann-reaction and against the customary views on phosphocreatine as a phosphagen.

INTRODUCTION

In the present study, we have examined to what extent the poisoning of an intact isolated frog sartorius muscle with iodoacetate[1] leads to an inhibition of two of its enzymes: creatine phosphoryltransferase, and phosphoglyceraldehyde dehydrogenase. With respect to the latter, it is the inhibition of this very enzyme which is regarded as the direct cause of the elimination of glycolysis[2], but it would be instructive to detect this action of the drug in living muscle by direct enzyme assay, also in order that it might serve as a basis of comparison for the other case examined. The main purpose of this work was to investigate creatine phosphoryl transferase, which *in vitro* is also sensitive to iodoacetate[3]. If this enzyme were also inhibited *in vivo*, one would be in great difficulty, as Ennor and Morrison[4] have recently brought up, to explain the decomposition of phosphoryl creatine in poisoned muscles. Even the recently discovered direct reaction between 1,3-diphosphoglycerate and creatine[5] might be of no avail since its enzyme is more sensitive toward iodoacetate than is creatine phosphoryltransferase.

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EXPERIMENTAL

Treatment of muscles

Sartorius muscles were dissected with the usual precautions from frogs (Rana pipiens, 3 in. body length, obtained in the fall and winter and kept at 3°). They averaged 33 mm *in situ* length and 90 to 100 mg net weight. After dissection, being tied with cotton threads at both ends, they were suspended in Ringer solution (120 ml for 1 to 2 muscles) and kept for 30 min well aerated at 0° with 95 % O_2, 5 % CO_2. Control muscles remained in this bath for the remainder of the experiment, while those to be poisoned received 12 mg (or other amounts) of Na- or K-iodoacetate and 0.2 mmoles of Na-lactate added to the bath. After 1 h in the cold, with constant aeration, the bathing solution was replaced by Ringer and the muscles left for a further 15 min. Both the control and the experimental muscles were then removed, blotted with tissue paper, frozen by immersion into isopentane at —180° and powdered. The powders were extracted with 25 volumes of 0.001 *M* KCl in 3 portions for 10 min each in the cold and centrifuged. Usually, one pair of muscles was employed for each experiment, since the extraction and fractionation procedures could be applied to as little as 100 mg of tissue.

Fractionation of enzymes

Creatine phosphoryltransferase, with the procedure employed, could not be determined directly in the extract without significant error, but this is reliably done after fractionation. The determination of triosephosphate dehydrogenase definitely requires separation from enzymes that would interfere with its assay. Specifically, incubation of the mixture with phosphoglyceraldehyde and DPN would lead to reoxidation of any DPN-H formed by the simultaneously present triosephosphate isomerase and glycerolphosphate dehydrogenase. We have, therefore, developed a microchemical separation procedure based upon the use of acetone[6]. This does not effect perfect separation of all enzymes concerned without overlap, but is sufficient to obtain the greatest part of the triosephosphate dehydrogenase without serious contamination.

We have also attempted to purify creatine phosphoryl transferase by application of the first steps of the procedure of NODA, KUBY AND LARDY, modified by the use of more dilute tissue extracts for complete extraction. This altered procedure gave very low yields, so that this modification cannot be tolerated.

For the acetone precipitation, the extract is kept in a bath at — 10° and acetone is gradually added first to 30 % (v/v) final concentration, and stirred for 30 min, whereupon the mixture is centrifuged in a graduated tube. Further additions of acetone are then made in the same manner until the required concentrations.

Determination of phosphoglyceraldehyde dehydrogenase

The assay was performed by measuring optically the reduction of DPN in the reaction:

ph. glyc. ald. + arsenate + DPN → DPN-H + arsenate + ph. glycerate

The test system was made up to the following composition: 1.6 ml 0.06 *M* cysteine in 0.03 *M* Na-pyrophosphate pH 8.4; 0.2 ml 0.003 *M* DPN; 0.1 ml 0.4 *M* Na-arsenate; enzyme and 0.03 *M* pyrophosphate to 2.9 ml; after 7 min standing, the reaction is initiated with 0.1 ml phosphoglyceraldehyde[8*]. In a mixed enzyme system containing phosphoglycerol dehydrogenase and triosephosphate isomerase, the interference due to the latter enzymes can reveal itself in various ways. When the contamination is moderate, the initial slope of the optical change would still be correct, but the reaction soon slows down and the validity of the initial slope cannot, in general, be asserted. We have once encountered a case in which the O.D. first went up and then down: this might be ascribed to contamination with a limited amount of isomerase and a greater amount of glycerol phosphate dehydrogenase. Fig. 1 gives an example of an assay of a fractionated extract from normal and from poisoned muscle. Such observations in various acetone precipitates (Fig. 2) indicate that the bulk of the phosphoglyceraldehyde dehydrogenase under our conditions precipitates without contamination between 32 and 38 % acetone, but that above 40 % acetone a sizable amount is precipitated in contaminated form; above 48 % acetone again, one of the contaminants seems greatly reduced. It is shown by Fig. 2 that in the case of poisoned muscles the bulk fraction of the enzyme is completely inhibited, or nearly so, and that no detectable activity occurred in the other fractions. The inhibition of the dehydrogenase has been complete when the muscle had been bathed for 1 h in the cold in 0.0005 *M* iodoacetate.

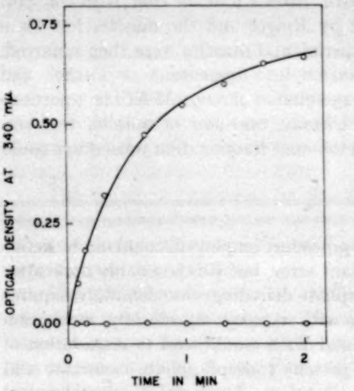

Fig. 1. Assay of phosphoglyceraldehyde dehydrogenase in fractionated muscle extract of control (top) and iodoacetate poisoned muscle (bottom curve). Composition of medium as indicated in the text; 0.5 ml of solution of the 32–38 % acetone fraction corresponding to 1.0 ml of original extract, was used.

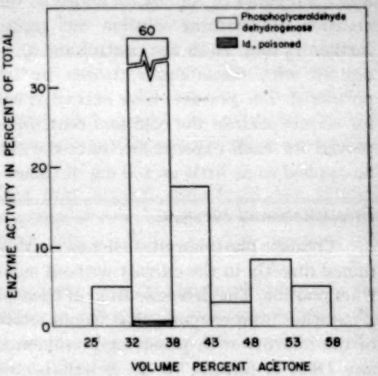

Fig. 2. Distribution of measurable phosphoglyceraldehyde dehydrogenase activity upon fractionation with acetone for control and iodoacetate poisoned muscle.

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[*] 21.6 mg DL-glyceraldehyde-1-bromide-3 phosphate dioxane are dissolved in 0.5 ml water, adjusted to pH 7 with 2 *N* NaOH, and diluted to 2 ml with water.

Determination of creatine phosphoryltransferase

In most cases, this enzyme was measured in the 38 to 60 % or the 38–70 % acetone fraction, by the reaction: $PC + ADP \rightarrow ATP + C$ executed at 37° in a mixture of the following composition: 2 ml glycine or glycylglycine buffer, 0.1 *M*, pH 7.2, with 0.05 *M* $MgCl_2$; 1 ml phosphocreatine, 0.002 *M*; 2 ml ADP, 0.001 *M*; enzyme and water to 8 ml (*cf.* ref.[3]). Samples were taken after various intervals, deproteinized with trichloroacetic acid at 0° neutralized with a small excess of NaOH, and analyzed for free creatine with diacetyl and α-naphtol. As is shown in Fig. 3, the enzyme from poisoned muscle was not inhibited, nor could significant inhibition be obtained even by dosages of the drug up to 0.002 *M* bathing solution per muscle.

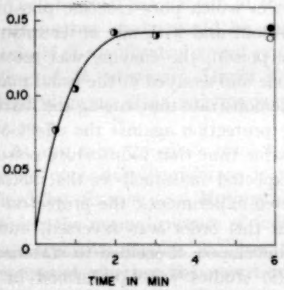

Fig. 3. Assay of creatine phosphoryltransferase in idoacetate poisoned (full circles) and control (open circles) muscle. Conditions of the experiment as given in the text.

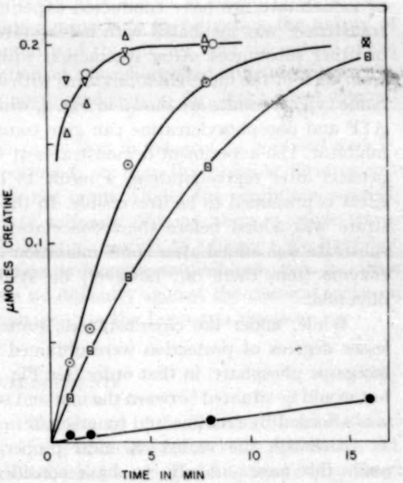

Fig. 4. Protection of creatine phosphoryl transferase against inhibition by iodoacetate. Purified enzyme, 0.4 mg, added to 5 ml of incubation mixture of the following composition: 0.001 *M* iodoacetate, 0.002 *M* KCl, 0.0004 *M* phosphocreatine or other substrate tested; pH 7.6. Incubation for 20 min at 0°, followed by addition of 15 ml acetone at —20°, stirring for 30 min. After centrifugation and washing, the precipitated enzyme is dissolved in 4 ml 0.002 % KCl, and 2 ml are used, in 16 ml of reaction mixture, for the enzymic assay as described for Fig. 3. Individual experiments refer to the inhibition by IAA as such (●) as compared to the unhibited control (○), the complete protection by PC (△) and ATP (△), the partial protection by AMP (⊙) and phosphate (⊡). Points obtained in the presence of arsenate or creatine were identical with the iodoacetate experiments without added substrates.

Inhibition of creatine phosphoryltransferase in vitro

The negative result just described made it desirable to test the susceptibility of the enzyme toward iodoacetate in solution. This has been done primarily with an enzyme preparation from frog muscle precipitated with acetone. At 10^{-4} *M* iodoacetate, this was inhibited about 35 %, while complete inhibition was found at 10^{-3} *M* concentration.

Similar figures were obtained with the enzyme from rabbit skeletal muscle (Fig. 4) where at 0.001 *M* iodoacetate, after 20 min interaction at 0°, the inhibition was not quite complete. These figures represent a somewhat lesser inhibition than was found by ENNOR AND ROSENBERG[3]. Undoubtedly, the conditions of the experiments were somewhat different in each of these cases. It is clear that the sensitivity of the transferase is very close to that of the dehydrogenase, and that it would not be possible to find a concentration of iodoacetate which would achieve complete poisoning of the triosephosphate dehydrogenase without at the same time effecting a significant inhibition of the phosphoryl transferase as well.

Protection of creatine phosphoryltransferase against iodoacetate inhibition

A difference between the *in vitro* and *in vivo* inhibition studies is that in the latter case the enzyme may be presumed to be in contact with some of its substrates. In order to test whether this circumstance might protect the enzyme against the action of iodoacetate, we have conducted experiments in which pure creatine phosphoryl transferase[7] was incubated with iodoacetate, without and with one of its substrates or other substances. After interaction with the poison, the enzyme was recovered from each of the incubation mixtures with acetone and assayed in the usual manner. Some typical results are shown in Fig. 4, which demonstrate that among the reactants ATP and phosphorylcreatine can give complete protection against the effect of the inhibitor. The experiment demonstrates at the same time that iodoacetate poisoning persists after reprecipitation, a result to be expected inasmuch as the inhibitory effect is presumed to be irreversible. In the quoted experiments, the protective substrate was added before the iodoacetate; when this order was reversed, and the substrate was added after some inhibition had developed, it seemed to stabilize the enzyme from there on; however, no systematic studies were performed in that direction.

While, under the circumstances, protection was complete with ATP and PC, lesser degrees of protection were obtained by ADP and pyrophosphate, AMP and inorganic phosphate, in that order (see Fig. 4, where ADP and PP data are omitted, but would be situated between the first and second curves from the left). No protection was afforded by creatine and by arsenate.

Although the varied chemical properties of the protective substrates would make this most unlikely, we have considered whether the substances in question might act by a direct chemical elimination of the iodoacetate added. This is contradicted by the fact (Fig. 4, legend) that iodoacetate can be present in stoichiometric excess, and also by the observation, not being described in detail, that triosephosphate dehydrogenase is not protected by these substances.

DISCUSSION

In the present study, a contribution has been made to the problem of assaying phosphoglyceraldehyde dehydrogenase in complex enzyme mixtures, and a direct demonstration has been brought of the classical view that in iodoacetate poisoning anaerobic glycolysis is stopped by virtue of a complete or nearly complete inhibition of this enzyme. This view was originally based upon the demonstration by MEYERHOF AND KIESSLING[2] that in fermenting muscle extracts the oxidation of phosphotriose is inhibited but the conversion of phosphoglycerate is unaffected by iodoacetate. Although this is good evidence, we believe it significant to set the precedent of detecting the *in vivo* inhibition by subsequent recovery and analysis of the enzyme, especially since our studies on the creatine enzyme show that the expected inhibition is absent in the intact muscle. Unfortunately, the principle of our procedure is only applicable to irreversible inhibitors.

The same muscles displayed no inhibition of creatine phosphoryl transferase, although it could be confirmed that this enzyme *in vitro* is nearly as sensitive toward iodoacetate as is the dehydrogenase. Hence, it is obvious that without special circumstances complete inhibition of the latter enzyme should be accompanied by marked inhibition of the former one as well. Among such special circumstances, one might think of compartmentalization of the cell; without wishing to exclude such a possibility, we feel that a satisfactory explanation of the discrepancy is offered by our finding that several substances, most effectively ATP and phosphorylcreatine themselves, cause the enzyme to be completely resistant against the action of iodoacetate.

The chemical mechanism of this protection is a matter of considerable interest, and its further study would be a suitable approach to investigating the nature of the role of the sulfhydryl group in the activity of the enzyme, and the nature of the binding of its substrates. Direct interaction of phosphorylated substrates with the sulfhydryl group of an enzyme is often postulated but, so far, lacks direct demonstration.

At any rate, the given results demonstrate that in iodoacetate poisoned muscle, creatine phosphoryltransferase is not poisoned, so that the possible dilemma recognized by ENNOR AND MORRISON[4] does not occur. Under the conditions studied, in which the normal picture of iodoacetate poisoning obtains, there is ample transphosphorylative enzyme to allow the Lohmann reaction to proceed. We have not investigated whether the new Cori-reaction is likewise uninhibited. But in either case, our study of these phenomena raises no difficulty against the classical views on the role of phosphorylcreatine as a phosphagen via the Lohmann reaction.

ACKNOWLEDGEMENTS

This investigation was supported by Grant No. H-3067 of the National Heart Institute of the National Institutes of Health, U.S. Public Health Service, Bethesda, Md.

P. P. worked as a visiting Research Biochemist at the Los Angeles County Heart Association Cardiovascular Research Laboratory, supported by a special Grant in Aid from the 167th District of the Rotary International (France) and the "Foundation Philippe" Paris.

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STUDIES ON THE GLUCONO-δ-LACTONASE OF *PSEUDOMONAS FLUORESCENS*

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(Received March 27th, 1959)

SUMMARY

The first stage of the oxidative metabolism of D-glucose by *Pseudomonas flucrescens* is the conversion of D-glucopyranose into D-glucono-δ-lactone. This lactone is hydrolysed to gluconic acid by an enzyme found in the soluble fraction of cell-free extracts of the bacterium. The lactonase has been purified a hundredfold and some of its properties determined. It is completely specific for D-glucono-δ-lactone and its apparent ability to hydrolyse D-glucono-γ-lactone is due to the spontaneous interconversion of the γ- and δ-lactones, followed by the enzymic hydrolysis of the latter. The apparent spontaneous hydrolysis of the γ-lactone below pH 7 follows a similar course. In the course of this work the mechanism of the interconversion of glucono-γ-lactone, glucono-δ-lactone and gluconate in the pH range of 4–8 has been determined.

INTRODUCTION

It is known that the oxidative metabolism of D-glucose by *Pseudomonas fluorescens* passes through D-gluconic acid and 2-keto-D-gluconic acid[1]. By analogy with the oxidative metabolism of D- and L-arabinose and D-galactose by *Pseudomonas saccharophila*, where the first intermediate produced from the sugar is a glycono-γ-lactone[2–4], the expected first intermediate in the oxidation of glucose by *P. fluorescens* is a gluconolactone. Since the rate of hydrolysis of D-galactono-γ-lactone and the arabono-γ-lactones is extremely low below pH 7, their conversion under physiological conditions to the corresponding acid requires the action of an enzyme. Such enzymes were indeed found in extracts of *P. saccharophila* but not further investigated[2–4]. In contrast, the spontaneous hydrolysis of both D-glucono-γ-lactone and D-gluconc-δ-lactone is quite rapid at pH 5–7. Nevertheless the rate does not appear to be great enough to allow the spontaneous hydrolysis of these lactones to be a step in a metabolic pathway. HERTLEIN[5] found that in fact an enzyme hydrolysing gluconolactones could be detected in the supernatant remaining after the particulate fraction containing the oxidative systems[1] had been centrifuged out of a preparation of sonically disrupted *P. fluorescens* cells.

Addition of this supernatant accelerated the spontaneous hydrolysis of both the

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γ- and δ-lactones. Since γ-lactone would be the oxidation product of D-glucofuranose and δ-lactone of D-glucopyranose, this observation could be interpreted in several ways. Two separate glucose dehydrogenases with their concomitant hydrolases might be present in *P. fluorescens*. If there were only a single oxidizing enzyme, then there might be two hydrolases present of which one was irrelevant to the main oxidative pathway. Alternatively there might be a single hydrolase with a specificity broad enough to include both lactones, one or both of which might be produced oxidatively. Finally there might be a single hydrolase with its specificity directed towards one of the lactones only, the apparent hydrolysis of the other being due to the complex equilibrium between lactones and gluconate known to exist in slightly acid solution.

The following experiments present the evidence that the last possibility is the correct one. Only a single lactonase is present, the true substrate of which is the δ-lactone, and the first step in the metabolism of glucose by *P. fluorescens* is the oxidation of D-glucopyranose to D-glucono-δ-lactone.

EXPERIMENTAL

Methods and materials

Lactones were determined by a slight modification of the method of HESTRIN[6]. O.D. was determined at 660 mμ in the Beckmann Spectrophotometer. The formation of bubbles was avoided by keeping the cuvettes in a very dilute solution of detergent, rinsing them in distilled water and wiping the external surfaces immediately before use. No way was found that would prevent the appearance of bubbles on the interior surfaces of the cuvettes if they were allowed to dry out before coming in contact with the sample.

Experiments on the spontaneous decomposition of lactones in various buffers were in general carried out by adding pre-heated buffer solution to a weighed amount of lactone in a pre-heated tube and withdrawing samples as required. An initial concentration of about 5 mM was found to be satisfactory for most experiments. The colour yield varied among the different lactones used[4] and where absolute values of concentration were required an appropriate standard colour yield had to be used. Unless otherwise stated all experiments on both the spontaneous and the enzymic hydrolysis of lactones were carried out at 30°.

The lactonase was assayed as follows: To a pre-heated mixture containing 2.5 ml of 0.5 M sodium acetate buffer of pH 5.5 and 0.25 ml of a suitably diluted enzyme solution was added 0.1 ml of a freshly-prepared glucono-δ-lactone solution containing 2.5 mg of the lactone. The blank was identical except that it contained 0.25 ml of water instead of the enzyme solution. Since the assay depends on the variation in the rate of a first-order reaction at a substrate concentration far above the MICHAELIS constant of the enzyme, neither the exact equivalence of the lactone concentration in the two tubes nor its absolute value was critical as long as it was approximately 5 mM. Samples were pipetted directly into the Hestrin alkaline hydroxylamine reagent at zero and 10 min. Enzyme concentration (units/ml) was defined as

$$100 \times \left(\log_{10} A^{\mathrm{Enz}}_{0'} - \log_{10} A^{\mathrm{Enz}}_{10'}\right) - \left(\log_{10} A^{\mathrm{Blank}}_{0'} - \log_{10} A^{\mathrm{Blank}}_{10'}\right),$$

A^{Enz} and A^{Blank} being the absorbencies given by samples from incubation mixtures with and without enzyme. The unit is thus determined by the increase, after the addition of enzyme, in the slope of the linear plot of log A against time which we find for the spontaneous hydrolysis of glucono-δ-lactone.

L-Gulono-γ-lactone, D-galactono-γ-lactone, D-glucuronolactone, D-glucono-δ-lactone, D-arabono-γ-lactone, D-ribono-γ-lactone and lactobiono-δ-lactone were commercial products agreeing in m.p. with reported values. D-Glucono-γ-lactone was prepared by Mr. B. C. HERTLEIN; both gluconolactones agreed in initial optical rotation with reported values[7]. D-Erythronolactone was the gift of Dr. C. E. BALLOU. Potassium D-gluconate was prepared from D-glucono-δ-lactone and various other potassium glyconates by hypoiodite oxidation[8]. All glyconates were crystallized twice from aqueous methanol before use; small samples of lactones in solution were prepared from them by boiling with 0.1 N HCl.

P. fluorescens was grown in the presence of glucose essentially as described by WOOD AND SCHWERDT[1]. The harvested cells were sonically disintegrated and cell debris and the particle fraction removed[5]. The supernatant, in pH 7.5 veronal–acetate buffer[9], could be stored indefinitely at refrigerator temperature without any great loss of lactonase activity. It contained on the average 4–6 mg of protein per ml by the LOWRY method[10] and about 2 units of lactonase per mg of protein. The activity differed among the samples, but its level could not be related in any systematic way to variations in the growth of the bacterium, or the subsequent treatment of the preparation.

Purification of the lactonase

The supernatant was tested for its power to accelerate the spontaneous hydrolysis of a variety of lactones. It proved inactive against D-galactono-γ-lactone, D-mannono-γ-lactone, L-gulono-γ-lactone, D-glucuronolactone, D-xylono-γ-lactone, D- and L-arabono-γ-lactones, D-lyxono-γ-lactone, D-ribono-γ-lactone, D-erythronolactone, lactobiono-δ-lactone, and metasaccharin. Since the hydrolysis of D-glucono-γ-lactone was soon seen to be kinetically complex, the δ-lactone was chosen as the assay substrate with occasional reference to the γ-lactone to check that there was no separation of the activities against the two lactones.

A large number of possible adsorbents were tested for their ability to adsorb the lactonase using a variety of buffers, pH ranges, and concentrations. Only kaolin and charcoal would adsorb the lactonase, and only the adsorption on kaolin from 0.1 M Tris–HCl at pH 7.5 was reversible. For this process to be efficient the bulk of the interfering protein more readily adsorbed on kaolin than the enzyme had to be removed. This was accomplished by fractionation with ammonium sulfate followed by adsorption of most of the protein in the active fractions on diethylaminoethyl-cellulose (DEAE-cellulose), which would not adsorb the enzyme under any practicable conditions.

In the final purification procedure the crude supernatant was fractionated at 45, 55 and 75 % saturation of ammonium sulfate at 0°. The bulk of the activity appeared in the 55–75 % fraction. A small amount of enzyme of almost unchanged specific activity appeared in the 45–55 % fraction, and this material was refractionated and the most active fraction added to the main fraction if there was enough activity to justify this procedure. The other fractions were discarded. The active fractions were redissolved in half the original volume of 0.1 M Tris–HCl, pH 7.5, and refractionated, after a preliminary centrifugation to remove insoluble material, at 50, 60 and 70 % saturation of ammonium sulfate at 0°. The bulk of the activity appeared in the 50–60 % fraction. If sufficient enzyme activity (often of a rather high specific activity) had been precipitated in the 60–70 % fraction it was bulked with the main fraction, otherwise it was discarded with the other less active fractions.

The active fraction was redissolved in 0.1 *M* Tris–HCl buffer, pH 7.5, and to it was added an equal volume of a gravity-packed slurry of diethylaminoethyl-cellulose, equilibrated with the same buffer. After 1 h in the cold room, the DEAE-cellulose was centrifuged off and the pellet washed with ¼ volume of buffer which was combined with the supernatant.

A small portion of this active supernatant was treated with successive portions of kaolin to determine the amounts necessary for the beginning and completion of enzyme adsorption. The point at which this adsorption began varied between total additions of 20 and 120 mg of kaolin/ml for different samples. Another 30 mg of kaolin/ml was usually enough to complete the adsorption. The main bulk was now treated successively with two lots of kaolin in amounts suggested by the test, the kaolin being shaken with the supernatant for 15 min and then centrifuged off. The second lot of kaolin with the adsorbed enzyme was washed with two further portions of the same buffer (no activity was removed) and the enzyme was then eluted from the kaolin with two successive portions of 0.5 *M* Tris–HCl buffer, pH 9.5. The combined eluates were adjusted to pH 7.5 and dialysed against the 0.1 *M* buffer. The active dialysate could be stored indefinitely in the refrigerator.

Table I summarizes a typical purification procedure for the enzyme and shows a better than 100-fold purification. The final solution had a protein content of about 0.1 mg/ml, no absorption in the visible region and a ratio of absorbencies at 260 and 280 mμ of 0.9. It still accelerated the hydrolysis of D-glucono-γ-lactone as well as that of the δ-lactone, but was inactive against all other sugar lactones.

TABLE I

PURIFICATION OF GLUCONOLACTONASE

Fraction	*Volume of solution ml*	*Units*	*Specific activity units/mg protein*	*Yield %*
Particle-free supernatant	350	4880	2.22	
First ammonium sulfate	150	3290	4.42	67
Second ammonium sulfate	31	2190	8.23	45
DEAE-cellulose treated	60	1990	36.6	41
Kaolin-adsorbed	25	1160	267	22

RESULTS

General properties of the lactonase

Preliminary experiments showed that, although the rate of spontaneous hydrolysis of D-glucono-δ-lactone below pH 7 was noticeably affected by the nature and concentration of the buffer that was used to hold the pH constant, there was no correlation between this effect and the influence of the same ions on lactonase activity. In particular the enzyme was strongly inhibited by phosphate ions, and most buffer ions showed some depressing effect over acetate (for the more acid end of the pH-activity range) and collidine (for the more basic). A composite pH-activity curve for these two buffers is shown in Fig. 1. Since acetate was the least inhibitory buffer found, it was used wherever possible for all further work on the enzyme; kinetic experiments on the spontaneous decomposition of lactones were mostly done in phosphate-free buffers, since otherwise it would be impossible to compare enzymic and spontaneous decomposition under identical conditions. The fact that fairly concentrated buffers had to be used to avoid sensible change in pH during the hydrolysis of the lactone meant that it was impossible to eliminate ionic effects in experiments with the enzyme, and no attempts will be made here to interpret comparative results under different conditions.

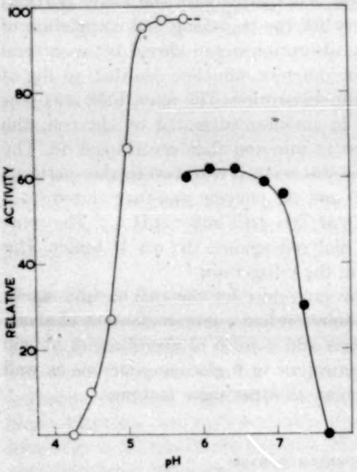

Fig. 1. pH-Activity curves for the enzymic hydrolysis of D-glucono-δ-lactone. ○, 0.5 M sodium acetate buffer; ●, 0.5 M collidine–HCl buffer.

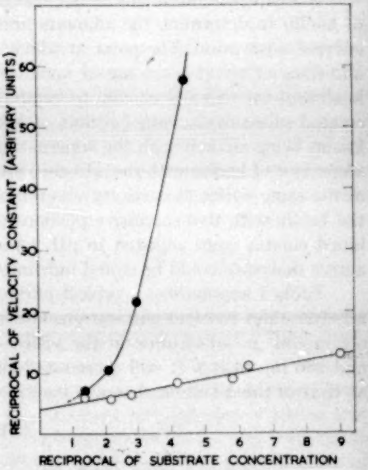

Fig. 2. Effect of substrate concentration on the enzymic hydrolysis of D-glucono-δ-lactone in 0.5 M sodium acetate buffer, pH 5.5. ○, alone; ●, in the presence of 0.033 M sodium phosphate, pH 5.5. Unit substrate concentration = $7.1 \cdot 10^{-3}$ M.

A determination of the MICHAELIS constant at pH 5.5 in 0.5 M sodium acetate buffer (standard assay conditions) gave $K_m = 1.8 \cdot 10^{-3}$ M. Fig. 2 shows the data on which this figure is based along with some other data for the phosphate inhibition of the enzyme. It is apparent that the inhibition cannot be diagnosed as competitive or non-competitive by the conventional criteria. A number of atypical curves similar to that in Fig. 2 were obtained for various concentrations of phosphate and arsenate. The concentration required for 50 % inhibition of the enzymic hydrolysis of 5 mM D-glucono-δ-lactone in 0.5 M acetate, pH 5.5, is approximately 50 mM phosphate or 10 mM arsenate. As the acetate concentration is lowered so are the inhibitory concentrations of phosphate and arsenate and even dilute buffers of phosphate alone are almost completely inhibitory.

It appeared that one way of eliminating unwanted ionic effects might be to use gluconate itself as the buffering species. Solutions of purified potassium gluconate, giving no initial reaction for lactone, and adjusted cautiously to the required pH with hydrochloric acid were used. At pH 5 the equilibrium lactone concentration in *M* gluconate was about 5 m*M*, in 0.1 *M* gluconate it was less than 1 m*M*. At pH 5.5 about 1 m*M* lactone was formed in *M* gluconate. These results were identical for solutions which were attained over several days at 30° or hastened by heating. Gluconate buffers should therefore have been usable for investigation of lactonase activity. These buffers were in fact completely inhibitory. Conditions at pH 5 and above in gluconate could be arranged so that there was a considerable measurable increase (first-order kinetics) in lactone over periods of 30–60 min at 30°. Under no conditions did the presence of enzyme have an effect in accelerating lactonisation, *i.e.* it did not catalyse the back reaction, at least in the presence of the excess gluconate which was necessary for any back reaction to occur at a pH where the enzyme would function.

A number of glyconates were added at a concentration of 50 m*M* to the standard assay mixture. The order of effectiveness was D-gluconate, D-xylonate (approximately 60% inhibition) > D-galactonate ≫ all others (zero inhibition within experimental errors). Further studies in this field would demand the synthesis in relatively pure form of other glycono-δ-lactones than the available glucono- and mannono-δ-lactones, since this evidence (inhibition of enzyme by excess product?) shows that D-galactono- and D-xylono-δ-lactones, never obtained except as mixtures in solution[11], might well be substrates.

Spontaneous hydrolysis of lactones

Preliminary experiments showed that the kinetics of spontaneous hydrolysis of D-glucono-γ-lactone were not first-order in the pH range 4–7. Nor could the observations be fitted to second or higher order kinetics. Since it was impossible to establish whether this lactone was in fact a substrate of the enzyme without understanding what occurred during the spontaneous hydrolysis, the hydrolysis of various glyconolactones by buffers of known pH was investigated. It is convenient to describe the results obtained under three heads.

Glyconolactones with no possibilities of isomerization (A). A number of lactones, *e.g.* D-erythronolactone and D-glucuronolactone, have the γ-lactone as their only possible structure. The kinetics of the spontaneous hydrolysis of these lactones is very simple. The plot of the logarithm of the amount of lactone remaining in solution against time elapsed since the beginning of the experiment is the straight line expected for first-order kinetics. Reaction velocity is thus proportional to lactone concentration. Fig. 3 shows some results for D-erythronolactone which can be taken as representative of all such first-order hydrolyses. When the values of k (first-order velocity constant) in 0.35 *M* Tris–maleate buffer were plotted against $[OH^-]$ for these two lactones, the two quantities were seen to be strictly proportional down to the lowest experimentally determinable reaction velocities (Fig. 4). The overall kinetics are thus simple second-order, with reaction velocity equal to k' [lactone] $[OH^-]$.

D-*glucono-δ-lactone* (B). In the pH range 4 to 8.5, the spontaneous hydrolysis of the lactone proceeds according to first-order kinetics. When k is plotted against $[OH^-]$, it is found that above pH 7.2 in 0.35 *M* Tris–maleate buffers, k is proportional to $[OH^-]$. Below pH 7, the value of k is much greater than that calculated by extrapolation from higher pH values. When other buffering systems are used it is found

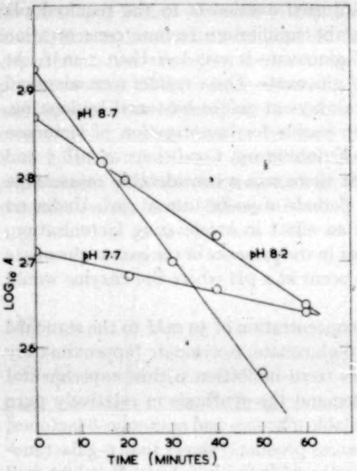

Fig. 3. Hydrolysis of D-erythronolactone in 0.35 *M* Tris–HCl buffers. *A* is the absorbency of a sample withdrawn and treated according to HESTRIN[6].

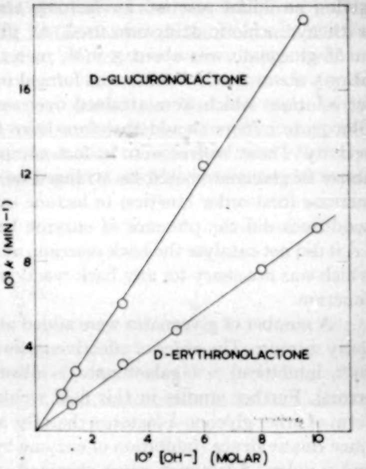

Fig. 4. Relation between hydroxyl ion concentration and first-order velocity constant for the hydrolysis in 0.35 *M* Tris–maleate buffer of two γ-lactones.

that results at pH 4 to 5 are hundreds of times greater than those calculated from the high-pH Tris–maleate data and *k* is so little influenced by pH that it is presumably not directly dependent on [H$^+$] or [OH$^-$]. Buffer influence is noticeable for the MCILVAINE (citrate–phosphate) buffer to at least pH 7.5. These results can be interpreted by supposing that the hydroxyl-ion catalysis is only a special case of a general negative-ion catalysis, the OH$^-$-dependent mechanism always becoming rate-determining at high enough pH. The basic data for these deductions is set out in Figs. 5 and 6.

It was impossible to show any mechanism other than the OH$^-$-dependent one for lactones of Class A, but below pH 7 their hydrolysis rates are negligibly low and any ionic effects would be less than experimental error.

Glycono-γ-lactones with possibilities of isomerization (C): Above pH 7.5 in 0.35 *M* Tris–maleate buffer the kinetics of the hydrolysis of L-gulono-γ-lactone, D-galactono-γ-lactone, D-xylono-γ-lactone and D-glucono-γ-lactone are identical with those of Classes A and B, being second-order with reaction velocity proportional to OH$^-$ and lactone concentration. Below this pH, D-galactono-γ-lactone is indistinguishable from Class A and its hydrolysis is negligibly slow below pH 7; the other lactones however, show quite different behaviour. As the pH falls, the hydrolysis at any given pH departs further and further from first-order kinetics. This is illustrated in Fig. 7 for D-glucono-γ-lactone and L-gulono-γ-lactone. These data could not be interpreted in terms of nth-order kinetics for any value of n. Characteristically there was a lag period, of increasing length as the pH was lowered, followed by a period of increasingly rapid hydrolysis. This might be simply explained by supposing that the observed overall

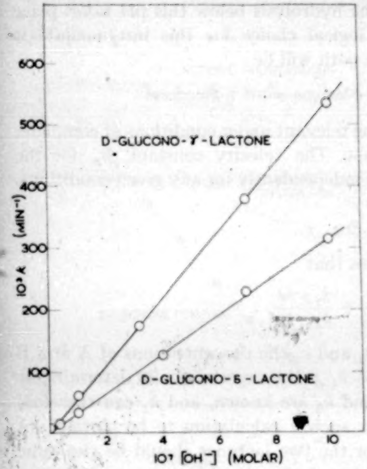

Fig. 5.

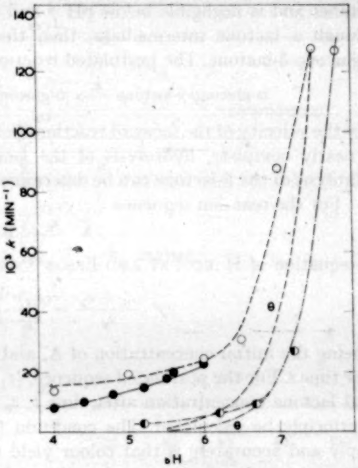

Fig. 6.

Fig. 5. Relation between hydroxyl ion concentration and first order velocity constant for the hydrolysis in 0.35 *M* Tris–maleate buffer of D-glucono-γ- and δ-lactones.

Fig. 6. Variation with pH in the first-order velocity constant for the hydrolysis of D-glucono-δ-lactone in various buffers. ◐, 0.35 *M* Tris-maleate; ●, 0.5 *M* sodium acetate; ○, McIlvaine (citrate–phosphate). θ = theoretical curves for a velocity constant proportional to hydroxyl ion concentration.

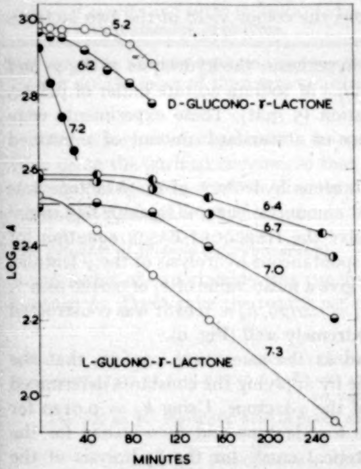

Fig. 7.

Fig. 7. Time course of the hydrolysis of D-glucono-γ-lactone and L-gulono-γ-lactone in 0.35 *M* Tris-maleate buffers. The curves for L-gulono-γ-lactone have been displaced 0.3 unit downward.

hydrolysis was the sum of consecutive processes, of which only the last resulted in actual destruction of lactone. This hypothesis was tested rigorously under the standard assay conditions for D-glucono-γ-lactone.

Spontaneous and enzymic hydrolysis of D-glucono-γ-lactone at pH 5.5

If the direct hydrolysis of D-glucono-γ-lactone follows the pattern of Class A lactones and is negligible below pH 7 and the hydrolysis below this pH takes place through a lactone intermediate, then the logical choice for this intermediate is D-glucono-δ-lactone. The postulated reaction path will be

$$\text{D-glucono-}\gamma\text{-lactone} \xrightarrow{k_1} \text{D-glucono-}\delta\text{-lactone} \xrightarrow{k_2} \text{D-gluconate}$$

only the velocity of the forward reactions being relevant under conditions of complete, or nearly complete, hydrolysis of the lactone. The velocity constant, k_2, for the hydrolysis of the δ-lactone can be determined independently for any given conditions.

For the reaction sequence

$$\text{A} \xrightarrow{k_1} \text{B} \xrightarrow{k_2} \text{C}$$

the equation of HARCOURT AND ESSON[12] states that

$$\frac{c_1 + c_2}{c_0} = \frac{k_2\, e^{-k_1 t}}{k_2 - k_1} + \frac{k_1\, e^{-k_2 t}}{k_1 - k_2},$$

c_0 being the initial concentration of A, and c_1 and c_2 the concentrations of A and B after time t. For the postulated sequence, $(c_1 + c_2)$ is the experimentally determinable total lactone concentration after time t, c_0 and k_2 are known, and k_1 can therefore in principle be calculated. The condition for such a calculation to be carried out simply and accurately is that colour yield for the two lactones should be the same within experimental error, so that the replacement of γ- by δ-lactone or vice versa at a constant total lactone concentration gives a constant colour yield. A series of experiments showed that under our conditions the colour yield of the two lactones did not differ by as much as 1 %.

In the experiments designed to test the hypothesis, the hydrolysis of the γ- and δ-lactones was followed a number of times in 0.5 *M* sodium acetate buffer of pH 5.5 at 30° at constant initial lactone concentration (5 m*M*). These experiments were carried out both in the presence and absence of a standard amount of a purified lactonase preparation.

Under these conditions, k_2 for the spontaneous hydrolysis of the δ-lactone was 0.0181 min^{-1}; in the presence of the standard amount of enzyme it was 0.043 min^{-1} (Fig. 8). A graphical method was used to solve the HARCOURT-ESSON equation for each experimentally determined point in the spontaneous hydrolysis of the γ-lactone. The values of k_1 so obtained were averaged to give a mean value of k_1 of 0.0120 min^{-1}. When the theoretical curve using the values $k_1 = 0.0120$, $k_2 = 0.0181$ was constructed it was found to fit the experimental points extremely well (Fig. 9).

It was possible to check this result and at the same time confirm that the δ-lactone was the true substrate of the enzyme by applying the constants determined above to the apparent enzymic hydrolysis of the γ-lactone. Using $k_1 = 0.0120$ for the spontaneous transformation of γ-lactone to δ-lactone and $k_2 = 0.043$ for the enzyme hydrolysis of the δ-lactone, a theoretical curve for the hydrolysis of the γ-lactone in the presence of the enzyme could be calculated. This curve was also found to fit the experimental points well (Fig. 9).

Since the two constants had been determined in experiments quite independent of the one whose results they were used to predict, it appears that the two hypotheses, (a) that the γ-lactone is transformed to the δ-lactone as the first step in its hydrolysis and (b) that the true substrate of the lactonase of *P. fluorescens* is D-glucono-δ-lactone, are confirmed.

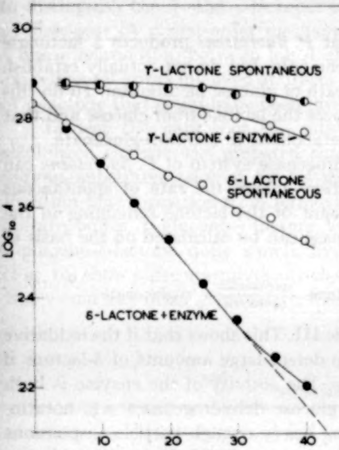

Fig. 8. Hydrolysis of D-glucono-γ- and δ-lactones in 0.5 *M* Na acetate buffer at pH 5.5 in the absence of enzyme and in the presence of identical amounts of enzyme.

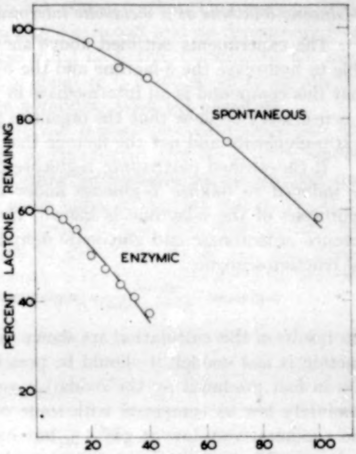

Fig. 9. Fitting of curves calculated on the basis of two sequential reactions to the experimental points for the apparent spontaneous and enzymic hydrolyses of D-glucono-γ-lactone at pH 5. The curve for the enzymic reaction has been displaced 40 units down the axis for clarity. The experimental points for the spontaneous reaction are the mean of three separate experiments and are those actually used in the calculation of k_1.

A further set of experiments using crude supernatant gave identical results to those using the purified enzyme, so that there is no evidence that an enzyme capable of hydrolysing the γ-lactone directly existed in the original preparation and was eliminated during the purification.

The kinetics of lactone interconversion

A series of experiments was run to determine the effect of pH on the velocity constant k_1. These gave the results set out in Table II.

TABLE II

EFFECT OF pH OF 0.5 *M* SODIUM ACETATE BUFFERS ON THE FIRST-ORDER VELOCITY CONSTANTS FOR THE HYDROLYSIS OF D-GLUCONO-δ-LACTONE AND THE TRANSFORMATION OF D-GLUCONO-γ-LACTONE TO THE δ-LACTONE

The pK at 30° for acetic acid is taken as 4.76 (ref. [13]) and pK_w at 30° as 13.83 (ref. [14]).

pH	k_2 *Velocity constant for the hydrolysis of the δ-lactone (min⁻¹)*	k_1 *Velocity constant for the transformation of the γ-lactone (min⁻¹)*	*[CH_3COO^-] (molar)*	*[OH^-] (molar × 10^{10})*
4.0	0.0084	0.0008	0.074	1.5
4.4	0.0105	0.0014	0.152	3.7
4.8	0.0125	0.0045	0.262	9.3
5.2	0.0148	0.0076	0.368	23.4
5.6	0.0200	0.013	0.436	58.9
6.0	0.0228	0.016	0.473	148

D-*Glucono-δ-lactone as a metabolic intermediate*

The experiments outlined above show that *P. fluorescens* produces a lactonase able to hydrolyse the δ-lactone and the δ-lactone only, but do not actually establish that this compound is an intermediate in the path of glucose metabolism. To do this it is necessary to show that the organism produces the lactone from glucose and that it is D-gluconate and not the lactone that is metabolised to 2-keto-D-gluconate.

If the washed particulate D-glucose dehydrogenase system of *P. fluorescens* can be induced to oxidize D-glucose under conditions where the rate of spontaneous hydrolysis of the δ-lactone is known, the amount of the lactone remaining in the absence of lactonase and gluconate dehydrogenase can be calculated on the basis of the reaction sequence:

$$\text{D-glucose} \xrightarrow[\text{dehydrogenase}]{\text{glucose}} \text{D-glucono-}\delta\text{-lactone} \xrightarrow[\text{hydrolysis}]{\text{spontaneous}} \text{D-gluconate}$$

The results of this calculation are shown in Table III. This shows that if the oxidative reaction is fast enough it should be possible to detect large amounts of δ-lactone if it is in fact produced by the oxidative enzyme. The activity of the enzyme is both absolutely low as compared with some other glucose dehydrogenases, *e.g.* notatin, and comparatively low at pH 5.5, but by using heavy enough particle suspensions it was possible to arrange conditions so that about 2 mmoles of glucose in 5 ml were oxidized in about 20 min. The lactone concentration determined at this stage was 0.23 m*M* instead of the theoretical 0.34 m*M*. Having regard to the large uncertainties in measuring such small amounts of lactone and the high concentration of particles with unknown adsorptive and residual enzyme activities, this result appeared to be adequate confirmation of the production of a lactone.

TABLE III

CALCULATED AMOUNTS OF D-GLUCONO-δ-LACTONE REMAINING AFTER THE OXIDATION OF D-GLUCOSE

Performed in 0.5 *M* sodium acetate buffer pH 5.5.

Time taken to oxidize one µmole of D-*glucose* min	D-*Glucono-δ-lactone remaining at the end of oxidation period* µmoles
1	0.98
10	0.90
20	0.83
50	0.65
100	0.46
200	0.27

To demonstrate that this lactone was in fact the δ-lactone, the experiment was carried out as before, but at the end of the reaction period a tightly fitting rubber cap pierced by inlet and outlet tubes was put over the end of the flask. Oxygen-free nitrogen was bubbled through the incubation mixture as rapidly as possible. The oxidative reaction was stopped in less than 1 min by this rapid removal of O_2 and the lactone present continued its spontaneous hydrolysis without further additions from glucose oxidation. The nitrogen bubbling was continued slowly and samples were withdrawn by pushing a syringe through the cap from time to time. The difference in hydrolysis rate and mechanism at pH 5.5 (Fig. 8) was quite large enough to distinguish the δ- and γ-lactones even in low concentration. Lactone was found to disappear by a first-order mechanism having $k \doteq 0.018$ and no divergency from a linear relationship between log A and time could be found. Considering the large experimental errors involved an upper limit of about 10 % could be set to the amount of γ-lactone that could have been present in admixture with the δ-lactone.

To test the nature of the substrate of the D-gluconate dehydrogenase, the solubilized enzyme was prepared according to HERTLEIN[5] and its activity against various substrates assayed by his method, using ferricyanide as an acceptor and Prussian blue formation from ferrocyanide as an index of enzyme action. In pH 4.3 acetate buffer, D-glucono-γ-lactone would be nearly stable over short periods and D-glucono-δ-lactone quite slowly hydrolysed to gluconate (Table II). The results (Fig. 10) show quite unequivocally that gluconate is the true substrate of the enzyme. Nor would any other glyconate tested act as a substrate.

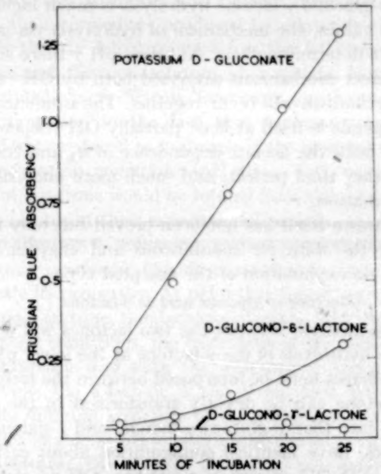

Fig. 10. Action of gluconate dehydrogenase on gluconate and gluconolactones in 0.6 M sodium acetate buffer, pH 4.3 at 30°. Substrate concentration 0.033 M.

DISCUSSION

The classical experiments[7,11] on the relative stability of lactones were carried out by making up an aqueous solution of a lactone and allowing hydrolysis and isomerization to proceed to a final equilibrium under constantly changing conditions of pH. Such experiments may give a very different picture of relative stability from ones carried out under constant conditions where the lactones are completely and rapidly hydrolysed (Table IV). Thus, in particular, D-glucono-γ-lactone which is much more rapidly hydrolysed than the δ-lactone at pH 7 to 8, will appear to be much more stable and to approach equilibrium much more slowly if the pH is allowed to fall uncontrolled below 5. The hydrolysis and isomerization of glyconolactones is obviously

TABLE IV

SECOND-ORDER VELOCITY CONSTANTS FOR THE HYDROLYSIS OF SOME GLYCONOLACTONES

The hydrolysis was carried out in 0.35 *M* Tris–maleate buffers.
Observed reaction rate = *k* [lactone] [OH⁻].

Lactone	*k* 10^3 × *litre·mol⁻¹ min⁻¹*
D-erythronolactone	10
D-glucuronlactone	21
D-galactono-γ-lactone	39
D-xylono-γ-lactone	64
L-gulono-γ-lactone	66
D-glucono-δ-lactone	310
D-glucono-γ-lactone	540

a very complex process and this study can do no more than fill in a few details for initial rates of hydrolysis under conditions far from equilibrium.

Since the rate of D-glucono-γ-lactone hydrolysis is much higher than that of the δ-lactone at higher pH values, the mechanism of hydrolysis via isomerization to the δ-lactone cannot be rate determining here. At about pH 7 there will exist a situation where hydrolysis by direct mechanisms catalysed both by OH⁻ and other ions and also by the indirect mechanism will occur together. The isomerization on which the indirect mechanism depends is itself at least partially OH⁻-dependent (Table II). It can be seen there that both the acetate-dependence of k_2 and the OH⁻-dependence of k_1 are suggestive rather than perfect, and much more analysis would be needed to resolve a complex situation.

The indirect mechanism itself has not been proved but only inferred as a satisfactory explanation of the data for spontaneous and enzymic hydrolysis of the γ-lactone. Nevertheless no explanation of the accepted type

$$\gamma\text{-lactone} \rightleftharpoons \text{gluconic acid} \rightleftharpoons \delta\text{-lactone}$$

with the acid as an intermediate between the two lactones will serve to explain the observed kinetics of the hydrolysis of the γ-lactone at the lower pH values and some intermediate or intermediates must be interposed between the lactone and gluconate. The idea that the γ-lactone can be directly transformed to the δ-lactone is made more attractive by the fact that D-glucono-γ-lactone and L-gulono-γ-lactone, which show anomalous kinetics, have identical configuration about carbon 4, along with *trans* disposition of the –CH$_2$OH on carbon 5 and the –OH on carbon 4 in the corresponding δ-lactones. D-Galactono-γ-lactone, with straightforward kinetics, has the opposite configuration, along with *cis* disposition of the groups in the δ-lactone. The possibility of the formation of any of the types of intermediates that might be postulated between the γ- and δ-lactones, with carbon 1 coupled to both carbons 4 and 5, would then depend on the overall stereochemistry of the molecule. It is possible to write at least one simple scheme for the OH⁻-dependent formation of such an intermediate (Fig. 11).

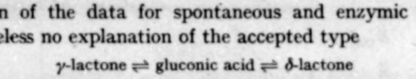

Fig. 11. A possible mechanism for the interconversion of D-glucono-γ- and δ-lactones.

In combination with the physico-chemical evidence, the biochemical evidence presented here shows that *P. fluorescens* produces enzymes capable of catalysing each step of the sequence

D-glucopyranose → D-glucono-δ-lactone → D-gluconate → products

although the possibility of the existence of other pathways of oxidative glucose metabolism is not eliminated. Similar enzymes have been reported from other sources but the interpretation of results concerned with their specificity is often dubious because the nature of the relationship between the γ- and δ-lactones in buffered solutions is more complex than the authors have realised. BENTLEY AND NEUBERGER[15] concluded that the primary product of the action of notatin (glucose dehydrogenase of *Aspergillus niger* or *Penicillium glaucum*) was D-glucono-δ-lactone by following the change in the optical rotation of the lactone in unbuffered solution, active notatin preparations producing very high concentrations of the lactone from glucose in a short time. STRECKER AND KORKES[16] and BRINK[17] produced evidence, falling short of final proof, that the intermediate produced by relatively much less active preparations of beef liver glucose dehydrogenase was the δ-lactone. The wider specificity of this last enzyme and the non-isolation of a specific lactonase made the exact definition of the metabolic path difficult. STRECKER AND KORKES found their greatest difficulties of interpretation in the fact that D-glucono-γ-lactone as well as the δ-lactone, appeared to be reduced to D-glucose in the reversed reaction of the enzyme. But under their incubation conditions (pH 7.0 → 6.7), our study shows that considerable amounts of δ-lactone would be formed from the γ-lactone even in the course of the few minutes (< 10) of their experimental period.

LIPMANN AND BRODIE[18], following earlier suggestions[19, 20] found δ-gluconolactonase in *Azotobacter vinelandii*, bakers' yeast and rat liver. They purified the yeast enzyme to investigate its properties. At pH 6 they found D-glucono-γ- and δ-lactones, 6-phospho-D-glucono-δ-lactone, D-galactono-γ-lactone and lactobiono-δ-lactone to be substrates. Under their conditions, the hydrolysis of the D-glucono-γ- and δ-lactones would be indistinguishable and since the γ-lactone is apparently hydrolysed more slowly by the enzyme, the δ-lactone is the *prima facie* true substrate. In view of the sterically totally different structure of the δ-lactones reported as substrates and D-galactono-γ-lactone (stable at pH 6), along with the wide variety of other γ-lactones reported as non-substrates, the enzymic hydrolysis of D-galactono-γ-lactone appears strikingly anomalous. Another important difference between the yeast and *P. fluorescens* lactonases is the non-inhibition of the yeast lactonase by phosphate, which was actually used as the buffering agent in the assay medium.

The induced enzymes of glucose oxidative metabolism in glucose-adapted *P. fluorescens* appear to be much more narrowly specific for D-glucose and its products than constitutive enzymes from other sources.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. M. DOUDOROFF, at whose suggestion and in whose laboratory this work was carried out. He is also indebted to Mrs. R. CONTOPOULOU for her valuable technical assistance and to Dr. M. CALVIN for his kind advice.

This work was supported, in part, by a grant from the National Institutes of Health (E-1808) and by State Cancer Research Funds of the University of California.

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SHORT-TIME $^{14}CO_2$ INCORPORATION EXPERIMENTS WITH SYNCHRONOUSLY GROWING *CHLORELLA* CELLS

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(Received March 20th, 1959)

SUMMARY

After a tenfold dilution of an initial culture, grown at 25° and 10,000 lux to a density of 18 ml packed cells per liter and containing a high percentage of small cells, cells of *Chlorella pyrenoidosa* have been grown synchronously at 20° and 12,000 lux. In 2- and 5-min photosynthesis experiments in the presence of $^{14}CO_2$, the incorporation of ^{14}C per unit volume of packed cells was increased in progressing stages of cell development. In later stages of the cell development prior to cell division, the portion of radioactivity in the 80% and 20% ethanol-insoluble material was higher than during the initial growth of the cells. At the same time a large increase of ^{14}C incorporated in sucrose and a decrease of ^{14}C incorporated in alanine have been observed.

INTRODUCTION

The different morphological and physiological changes occurring in the development of individual cells must be accompanied by shifts in their metabolic patterns. Synchronously growing cultures of unicellular organisms offer an advantageous material for studies of the metabolic processes characteristic of different phases of cell life. Some work along these lines, using *Chlorella* as test organisms, has been published by TAMIYA and co-workers[1-7]. They have developed a technique for the synchronous growth of *Chlorella* and have used it as a basis for biochemical studies of developmental processes. A different technique to induce synchronization in *Chlorella* cultures has been used by SOROKIN AND MYERS[8,9] and LORENZEN AND PIRSON[10-12].

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In this laboratory numerous studies have been made of the distribution of $^{14}CO_2$ after photosynthesis in populations of *Chlorella* heterogeneous with respect to the stages of cell development[13]. The experiments reported here were designed to study the distribution of $^{14}CO_2$ after short periods of photosynthesis (2 to 5 min) in relatively uniform cell populations of *Chlorella* at different stages of cell development. Differences in metabolic processes characteristic of different states in the development of the *Chlorella* cell have been found. Differences in the rates of incorporations of $^{14}CO_2$ and its resulting distribution were observed. Observations on the technique of growing *Chlorella* cells synchronously are made.

METHODS

Synchronous growth

The conditions described by TAMIYA *et al.*[1-4], adapted to the culture equipment used in our laboratory for the usual *Chlorella* cultures, were followed to induce synchronous growth of the algae. The algae were grown in the continuous-culture apparatus described by HOLM-HANSEN *et al.*[14]. It consists of a temperature-controlled column containing about 1000 ml of algal suspension, which is aerated with 4 % CO_2 in air. The column itself is a double-walled tube with the algae in the outer layer, which has a thickness of about 0.75 cm, while the inner tube contains circulating water to maintain any desired temperature. The tube is surrounded by a bank of eight 20-W fluorescent lights yielding a constant light intensity. The light intensity can be adjusted by varying the distance of the lights from the tube. The automatic dilution of the culture with fresh nutrient solution to maintain a constant density of algae was eliminated. Instead, new nutrient solution was supplied manually at the appropriate times from a reserve flask.

The culture of *Chlorella pyrenoidosa* was started by inoculating the tube with a sample of cells from a stock culture to give an initial algae population of 0.5 to 1.0 ml wet packed cells/l. The culture medium had the following composition: KNO_3, 5.0 g; $MgSO_4 \cdot 7\,H_2O$, 2.5 g; KH_2PO_4, 1.1 g; $Ca(NO_3)_2$, 0.02 g; EDTA[15], 1 ml; Hoagland's "A5" solution, 1 ml; distilled water to make up 1 liter[2,16].

The culture was grown at a temperature of 25° and a light intensity of 10,000 lux for 4 to 5 days. At that time the algae population had a density of about 18 ml packed cells/l and contained a high percentage of cells with a small cell diameter. Several initial experiments had shown that the percentage of small cells in the population increases with increasing density of the culture. The lights were then turned off and the culture left in the dark for 2 days.

The synchronous growth of the culture was initiated by diluting 100 ml of this *Chlorella* suspension with 900 ml of nutrient solution and growing at 20° with a light intensity of 12,000 lux. This time of growth initiation is defined as "0 h" and the subsequent stages of synchronous growth are designated by the time interval from this dilution. In initial experiments a temperature of 15° was used, following TAMIYA's conditions. However, this low temperature was not satisfactory with our *Chlorella* strain and under our experimental conditions for *continuous* synchronous growth of the culture; even though the first cycle after induction seemed to be unaffected, upon further growth of the culture at 15° the cell contents appeared granulated at the second or third cell-division cycle. This was caused by fat droplets accumulating in the cells. In addition, the growth and division of the cells were inhibited. The effect of the low temperature could be reversed by increasing the temperature to 25°. At 20° these effects were not observed. However, by reduction of the temperature to 20° a sufficient slowing down of the cell growth was obtained to facilitate the observation and sampling of *Chlorella* cells in relatively uniform stages of the growth-and-development cycle.

Under the above conditions one cell cycle extended over a period of about 36 h, during which time the number of cells, the sizes of cells, and the volume of packed cells have been determined. This period is characterized by a gradual increase of the average cell diameter and of the volume of packed cells per unit volume of the culture, and by a constant number of cells per unit volume of the culture. The best description of the stages of the synchronously growing culture is given in the variation curves for the cell diameter. Figs. 1a, b, and c represent the variation curves obtained for the cell cultures used for the short-time photosynthesis experiments described below. For each of these curves the diameters of 200 cells were measured in a microscope with an ocular micrometer. The smallest cells, derived from the cell division, usually show ellipsoidal shapes. No choice for the direction of diameter was made; the cells were measured in the direction in which they happened to lie in the scale of the ocular micrometer.

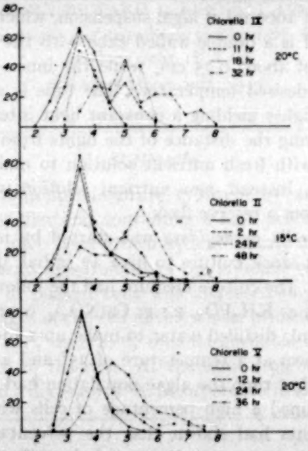

Fig. 1 a–c. Variation Curves of cell diameters (microns).

36 h after synchronous growth was initiated (by dilution and turning on the light) the culture contained a high percentage of dividing cells. If it is desired to obtain an additional cycle of synchronous growth, the light should be turned off at this time. An appropriate initial stage for further synchronous growth is thus obtained since under these conditions the cells present can not grow further. Synchronous growth may again be started after a dark period of 36 h at 25° by dilution and illumination as was done at "0 h".

$^{14}CO_2$ incorporation experiments

Samples were removed from the synchronously growing *Chlorella* culture at several time intervals (thus at different stages of cell development) to determine possible differences in the photosynthetic fixation of $^{14}CO_2$. The cells were centrifuged and rediluted to a 1% wet packed cell suspension by adding a diluted nutrient solution (1 part nutrient solution and 4 parts distilled water). (2 expts. with undiluted nutrient solution gave essentially the same results, but only a small portion of the final extract could be used for the analysis by paper chromatography.)

For these experiments the apparatus described by MOSES AND CALVIN[17] was used. It consists of cylindrical glass vessels with a flat bottom, of about 3.5 cm internal diameter, in which 1 ml of algae suspension forms a layer about 1 mm deep. The lid of the vessel is provided with an inlet and an outlet tube, the former reaching nearly to the bottom of the vessel. These vessels are arranged in a water bath at a temperature of 20° over a bank of eight 6-W fluorescent tubes yielding a light intensity of 10,000 to 20,000 lux at the bottom of the vessel. For a period of adaption of 30 min the 1-ml algae suspension is shaken in the light, while moistened air plus 1% CO_2 is blown over its surface. Immediately before addition of the radioactive carbon dioxide, the tube supplying the air plus 1% CO_2 was disconnected, 100 µl of $NaH^{14}CO_3$ (0.026 *M*, 40 µC) was injected into the suspension, and after 2 or 5 min respectively the cells were killed by adding 4 ml boiling 100% ethanol[18]. Generally duplicate experiments were done.

Analysis of extracts

After extraction with the 80% ethanol resulting from the killing procedure which removed amino acids, organic acids, free sugars, pigments, and lipids, the cells were extracted with 20% ethanol for 5 min at 50°, to extract mainly sugar phosphates. The volumes of the initial total suspension, the 80% ethanol extract, and the 20% ethanol extract were determined and samples of each with a drop of acetic acid were dried onto aluminum planchettes and counted with a Nuclear Chicago D 47 thin-window counter with automatic sample changer.

Aliquots of the combined 80% and 20% ethanol extracts were analyzed by paper chromatography. All chromatograms were made on oxalic acid-washed Whatman No. 4 filter paper, and were developed in phenol–water in the first dimension and in *n*-butanol–propionic acid–water in the second dimension[19]. Radioactive substances were located by exposure of the chromatograms to Dupont X-ray film No. 507E. Substances were identified by their chromatographic position, by cochromatography with known markers, and by reactions with appropriate sprays. They were counted directly from paper chromatograms with a Mylar-window Scott-type Geiger-Muller tube connected to a scaler.

RESULTS

The results of one of these experiments (*Chlorella* IX) in which samples were taken at the 11-h, 18-h, and 32-h stages, are presented in Tables I and II. The corresponding variation curves for the cell diameter in the synchronously growing culture are given in Fig. 1a. At the 11-h stage the first increase in the diameter of the cells can be seen. This increase in cell size is pronounced at the 18-h stage. The 32-h stage precedes the onset of cell division in the culture. Several other experiments gave comparable results.

Table I shows that the total amount of ^{14}C fixation per unit volume of cells more than doubled with the progressing cell development. The fixation of $^{14}CO_2$ reached the highest rate immediately before cell division occurred. At the same time the proportion of radioactivity appearing in the 80 % ethanol- and 20 % ethanol-soluble material decreased. This is particularly evident in the 5-min photosynthesis experiments, where the percentage of radioactivity in the soluble material is reduced by one-fifth.

TABLE I

INCORPORATION OF ^{14}C AFTER 2-min AND 5-min PHOTOSYNTHESIS

Incorporated with 40 μC of $NaH^{14}CO_3$ in different stages of cell development of synchronously growing *Chlorella* cells. (*Chlorella* IX) (total activity fixed by 1 ml of 1 % cell suspension).

Stage of Synchronous growth	*2-min photosynthesis*				*5-min photosynthesis*			
	Total activity (counts/min × 10^{-3})	*% of total activity*			*Total activity (counts/min × 10^{-3})*	*% of total activity*		
		80 % ethanol	*20 % ethanol*	*80 + 20 % ethanol*		*80 % ethanol*	*20 % ethanol*	*80 + 20 % ethanol*
11 h	1115	50.2	30.9	81.0	1891	56.3	20.2	76.5
	1167	47.0	36.0	83.0				
18 h	1739	56.1	26.6	82.7	2503	56.6	16.0	72.6
	1394	57.5	26.5	84.0				
32 h	3013	45.7	24.1	69.9	3901	49.4	11.3	60.6
	2707	50.3	28.3	78.7				

The pattern of ^{14}C incorporation shows great differences in the different stages of the cell development (Table II). The most striking differences are found for sucrose, for which the percentage of incorporation of $^{14}CO_2$ increases considerably in the 32-h stage (immediately before cell division), and for alanine, which shows a much higher proportion of incorporation at 11-h and 18 h than at 32 h. These differences in the amount of incorporation of ^{14}C in sucrose and alanine are found in both the 2-min and 5-min photosynthesis experiments. In the 2-min experiment, parallel to the decrease in the incorporation of ^{14}C in alanine at the 32-h stage in comparison with the 18-h stage, there can be observed a decrease for other amino acids, such as threonine, serine, glycine, aspartic acid, citrulline, and tyrosine. These characteristic differences are observed only with cultures showing normal cell-size variations with synchronous growth and not showing abnormalities such as granulation and fat deposition.

The same changes in the pattern of ^{14}C incorporation in different stages of the

TABLE II

INCORPORATION PATTERN OF ^{14}C AFTER 2-min AND 5-min PHOTOSYNTHESIS

Incorporated with $NaH^{14}CO_3$ in different stages of cell development of synchronously growing *Chlorella* cells (*Chlorella* IX). (% distribution of radioactivity on paper chromatograms*).

Spot No.	*Substance*	*2-min photosynthesis*			*5-min photosynthesis*		
		11 h	*18 h*	*32 h*	*11 h*	*18 h*	*32 h*
1	Origin	0.45	0.43	1.65	0.31	0.39	1.14
2	Diphosphates	8.50	5.88	6.11	4.46	3.34	2.81
3	Uridine diphosphoglucose	2.94	3.59	4.19	2.22	3.61	2.52
4	Monophosphates	30.62	23.97	33.52	14.18	17.76	16.32
5	Phosphoglyceric acid	9.43	9.74	10.30	4.96	6.73	5.89
6	P-Glycolic acid	0.71	0.64	0.51	0.26	0.44	0.29
7	Oligosaccharide	2.47	3.08	4.64	3.66	3.77	2.79
8	Maltose	0.39	0.58	0.59	0.51	0.61	0.49
9	Sucrose	*0.22*	*0.77*	*5.73*	*1.84*	*2.31*	*12.59*
10	Glucose	0.67	0.73	0.38	0.86	0.86	0.82
11	Fructose	—	—	0.53	1.19	0.64	0.25
12	Threonine	1.09	1.01	0.60	1.06	1.15	0.88
13	Serine	3.45	6.57	3.73	4.84	4.81	4.91
14	Glycine	0.43	1.23	0.64	0.56	0.65	0.40
15	Glutamic acid	3.12	4.75	4.94	8.56	12.04	15.43
16	Aspartic acid	9.54	10.23	3.98	15.71	10.09	11.64
17	Citric acid	0.50	0.98	0.69	0.61	0.94	0.99
18	Malic acid	6.15	8.01	7.72	6.55	5.70	8.60
19	Citrulline	0.77	0.44	0.28	0.37	0.31	0.31
20	Alanine	*14.09*	*12.25*	*4.10*	*21.02*	*16.36*	*6.45*
21	Tyrosine	0.44	0.90	0.50	0.91	0.89	0.59
22	Glutamine	0.35	0.34	0.73	1.36	1.19	1.06
23	Glycolic acid	1.76	1.68	1.75	1.10	1.29	1.11
	4–6 unknown spots	1.89	2.01	2.20	2.76	4.15	1.68

*** This list contains only spots on the paper which had been covered by the film. Some substances, *e.g.* valine and leucine, under the conditions used for the paper chromatographic separation run near to the border of the paper and have not been counted.**

TABLE III

INCORPORATION OF ^{14}C INTO SUCROSE AND ALANINE AFTER 2-min PHOTOSYNTHESIS

Incorporated with $NaH^{14}CO_3$ in different stages of cell development of synchronously growing *Chlorella* cells. (counts/min of sucrose and alanine spots on paper chromatograms*).

Experiment	*Stage of synchronous growth*	*Sucrose*	*Alanine*	*Ratio* $\frac{Sucrose}{Alanine}$
***Chlorella* II, 15°**	2 h	1069	1650	0.65
	24 h	1210	5300	0.23
	48 h	4491	764	5.88
***Chlorella* V, 20°**	12 h	1119	3248	0.34
	24 h	1404	4144	0.34
	36 h	2717	871	3.12

*** In both experiments samples with the same number of cells but increasing volume of packed cells have been used. Under these conditions we did not find the increase of ^{14}C fixation per unit volume of cells reported in Table I. Under the experimental conditions used, increasing volumes of packed cells had an influence on total fixation per unit volume of cells at the same stage of synchronous growth.**

cell development have been observed in repetitions of this experiment. The amount of radioactivity found in the sucrose and alanine spots of 2 further experiments are represented in Table III. The change in the incorporation pattern is expressed in a striking increase of the ratio sucrose: alanine.

DISCUSSION

Cultures of unicellular organisms growing under constant conditions usually contain cells at all stages of the cell life cycle. To obtain cells growing synchronously special treatments must be used, which will cause all cells to grow and divide at the same time. These treatments usually involve the application to the culture of conditions that enhance, slow down, or arrest the development of the cells or of portions of the cells in special stages of their life cycle. Exposure to optimal conditions initiates the synchronous growth of the culture. The first phase of this synchronized growth is a phase of recovery from the imposed stress and unbalance[20]. The contribution of such recovery process to differences observed in different stages of synchronous growth is difficult to determine. This difficulty constitutes a certain restriction on the use of synchronized cultures in studies on metabolic changes during the life cycle of the cell. The different methods for inducing synchronous growth differ in this respect only slightly. In the method of synchronization of *Chlorella* used by TAMIYA and co-workers[1–7] the prior treatment causes an exhaustion of energy or some substrate necessary for cell growth. SOROKIN AND MYERS[8,9] and LORENZEN AND PIRSON[10–12] used a periodic treatment of intermittent illumination. In their procedure, synchronization is lost without continuously repeated treatment, which shows that at least a portion of the cells is influenced by the period imposed.

TAMIYA *et al.* induced the synchronization of *Chlorella* cells by an initial culture phase of about 1 week at a reduced light intensity of 400 or 800 lux. They observed that the mass increase of the small *Chlorella* cells derived from cell division is dependent on a sufficient supply of light. Therefore, the method for induction of synchronization may be dependent upon the inhibition of cell growth by light reduction. In our experiments it was observed that a culture with a high percentage of small cells could be obtained by growing the initial culture in a light intensity of 10,000 lux to high cell density, in which case the light intensity per cell is small. Under these conditions the factors restricting the growth of the cells may be either a reduced effective light intensity or deficiency for some nutrient element in the culture.

With exception of some preliminary experiments, the first sample for the photosynthesis experiments with $^{14}CO_2$ was taken from the synchronously growing culture 11 to 12 h after applying optimal conditions. It is assumed that at this time the recovery processes have been finished. This is even more probable for the stages at 18 to 24 h and 32 to 36 h, between which the comparison of the results is mostly made.

In their investigations using synchronously growing *Chlorella* cultures TAMIYA and co-workers[1–7] found profound changes in different stages of cell life. The rate of light-saturated photosynthesis constantly decreased during the life cycle irrespective of whether the rate was referred to a unit of dry wt. of cells or to a unit number of cells. During the period of their observations (28 h in the light) the average cell volume increased about 3.5 times, while the rate of photosynthesis per cell decreased more than twice for O_2 production and more than 6 times for CO_2 consumption. This was reflected in a change of photosynthetic quotient (O_2/CO_2), which was around unity at the beginning of the development and reached a value of 3.3 in the large cells prior to cell division. The relative chlorophyll content was highest at the beginning of the life cycle and decreased during the increase in cell mass. The respiratory activity per dry wt. of cells was found to increase during the course of the development to about 2.5 times the initial value.

In his experiments with synchronously growing cultures of a high-temperature strain of *Chlorella pyrenoidosa* under diurnally intermittent illumination (9 h light, 15 h darkness) SOROKIN[9] observed during the first 3 to 4 h of cell growth in the light an increase of the rate of apparent photosynthesis referred to volume of packed cells, followed by a decrease during the further course of cell development. Referred to a single cell, the rate of the increase in the photosynthetic activity during the first 3 h of cell development is twice as high as the increase in the photosynthetic activity of the cell at the later stages of development, between 5 and 8 h. The disagreements between these results and the results of TAMIYA *et al.*[1,2] are explained by the observation that a change in temperature from the conditions of the culture to those of the manometric experiment (15° to 25°), as in the experiments of TAMIYA *et al.*, may greatly affect the results of measurements of the rate of photosynthesis.

From their studies on the rate of both endogenous and glucose respiration SOROKIN AND MYERS[8] concluded that the respiration rate rises immediately after the start of the development of a cell, soon (under their conditions after about 1 h) reaches a maximum, and undergoes a slow decline until the mother cell ruptures into daughter cells. Direct activation of respiratory enzymes by light could not be excluded as contributing to the initial rise of respiratory activity. Striking differences were observed in the shape of the curve for the rate of endogenous respiration, dependent on the time interval between harvesting cells and taking manometric readings.

Further important changes in the metabolism of cells in different development stages have been reported by IWAMURA[4], NIHEI[5,6] and HASE *et al.*[7]. The content of ribonucleic acid in per cent of dry wt. was found to be relatively constant throughout the whole course of cell development. In contrast, the percentage of desoxyribonucleic acid decreased more or less in the growing phase, and increased markedly in the "sporulation phase", prior to division of mother cells into a number of autospores. The stage immediately prior to nuclear division was further characterized by a light-induced O_2 evolution in the presence of phosphate and the absence of CO_2, and a corresponding phosphate fixation probably in the form of metaphosphate. The content of crude protein (in percentage of dry wt.) remained almost constant throughout the life cycle. A slight increase for total carbohydrate (in percentage of dry wt.) has been observed in later stages of the development; the portion of sucrose seems to have been significantly increased in the stage prior to cell division.

In the short-time ^{14}C-incorporation experiments described in this paper, an increasing incorporation referred to volume of packed cells with progressing development of the cell was found. Moreover, in the later stages of cell development, shifts in the incorporation pattern have been observed. The percentage of radioactivity in the 80 % and 20 % ethanol-insoluble material and the incorporation of ^{14}C in sucrose, mainly at the expense of incorporation in alanine, have been increased. These last results show some parallelism to the results of HASE *et al.*[7], who found differences in carbohydrate content during the life cycle. Their results also emphasize, in agreement with the results of Tamiya and co-workers, that important metabolic changes take place in the stages prior to cell division. The synthesis of proteins during the increase in cell mass, revealed by a high percentage of incorporation of ^{14}C in alanine, is replaced in these stages by other synthetic processes, including carbohydrate synthesis, and—according to the results of Iwamura[4]—synthesis of desoxyribonucleic acid.

Similar changes, as far as the short-time incorporation of ^{14}C in sucrose is concerned, have been observed in regenerating cells of the liverwort *Riella* (Stange, unpublished results), a cell material preparing for cell division and comparable in this respect with the *Chlorella* cells immediately prior to cell division. Like the *Chlorella* cells in respect to desoxyribonucleic acid, the regenerating cells of *Riella* have been shown to synthesize nucleic acids at the same time as carbohydrates[21].

The production of carbohydrates at this stage of development may be understood as an accumulation of reserve substances, as in higher plants at the transition from the vegetative to the reproductive phase, and (or) as a preparation of material for the new cell walls. The mechanism for the shifts in the metabolic pattern is unknown.

Further analysis of these processes would require experiments with longer incorporation times and with further examination of the insoluble material of the cell.

ACKNOWLEDGEMENTS

This work was sponsored in part by the U.S. Atomic Energy Commission and in part by the Department of Chemistry, University of California, Berkeley, Cali .

L.S. is research Fellow sponsored by Deutsche Bundesministerium für Atomkernenergie und Wasserwirtschaft (1957–58).

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THE β-GALACTOSIDASE OF MOUSE STRAIN L-CELLS AND MOUSE ORGANS

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(Received March 16th, 1959)

SUMMARY

The properties and distribution of the β-galactosidase of mouse strain L-cells and various mouse organs and tumors have been investigated. β-Galactosidase activity was found in extracts of strain L-cells and of all mouse organs examined. By the criteria which we have employed, the enzyme in all extracts appears to be the same molecular species.

Whereas β-galactosidase in crude preparations has a pH optimum of 2.8, the purified enzyme has a pH optimum of 2.2. The MICHAELIS constant using *o*-nitrophenyl-β-D-galactoside as substrate is approximately $7.0 \cdot 10^{-4}$ *M*. Galactose and lactose competitively inhibit the hydrolysis of *o*-nitrophenyl-β-D-galactoside by the enzyme; at 10^{-1} *M* concentration, glucose and melibiose are not inhibitory. Lactose is hydrolysed at approximately one-third the rate at which *o*-nitrophenyl-β-D-galactoside is hydrolysed. Specific antiserum precipitates, but does not neutralize the β-galactosidase.

The concentration of the enzyme differs widely from organ to organ. The highest specific activity is found in mouse embryonic and suckling mouse small intestine: adult small intestine shows low specific activity.

The significance of these findings is discussed.

INTRODUCTION

Biochemical diversity forms the basis of ontogeny in the multicellular organism. Such diversity should be reflected in qualitative and quantitative enzymic differences among the cells and organs of which the organism is composed. This communication describes the occurrence of the enzyme β-galactosidase in 13 mouse organs and tumors and mouse strain L-cells. The choice of β-galactosidase for this study was suggested by the availability of a convenient and extremely sensitive assay procedure for this enzyme.

MATERIALS AND METHODS

Preparation of strain L-cell extract

Tissue culture mouse strain L-cells derived from a single cell by SANFORD, EARLE AND LIKELY[1] were maintained by serial transfer in TC Medium No. 199 (Difco) supplemented with 10% horse serum. The cells were grown in suspension in medium 199 from a starting inoculum of about $3 \cdot 10^4$ cells/ml to a final concn. of 7–$8 \cdot 10^5$ cells/ml. "Bellco" (Bellco Glass Inc., Vineland, New Jersey) flasks equipped with a magnetic stirrer were used for growing the suspension cultures.

The cells were harvested by low-speed centrifugation, washed once in EARLE's saline, and resuspended in distilled water to a final concn. of 1–$2 \cdot 10^7$ cells/ml. The cells were then disrupted by sonic oscillation for 5 min in a Raytheon 10 kc Sonic Oscillator. The extract was cleared of cellular debris by centrifugation at 34,800 × g for 30 min at 4°.

Preparation of mouse organ extracts

Swiss strain white mice were used. Mouse embryos were obtained at about the 18th to 20th day of gestation. The mice were killed with ether and the organs removed, frozen, and then minced. The minced organs were suspended in distilled water and further disrupted in the sonic oscillator for 30 min. Following centrifugation at 34,800 × g for 30 min at 4°, a clear supernatant fluid containing between 10,000 and 20,000 μg of protein/ml was obtained.

β-galactosidase activity was found only in the supernatant fluid of the strain L-cell and organ extracts.

Purification of mouse intestinal β-galactosidase

The small intestines of 168 suckling mice (1- and 2-day old) were pooled and extracted as described above. All operations were performed at 4°. The nucleoproteins were precipitated from the extract with 0.01 *M* streptomycin sulfate. The supernatant fluid was dialysed against increasing concentrations of $(NH_4)_2SO_4$ until most of the β-galactosidase activity could be recovered in the precipitate; the enzyme was fully precipitated by $(NH_4)_2SO_4$ at 50% saturation.

Determination of β-galactosidase activity and protein

In experiments using *o*-nitrophenyl-β-D-galactoside (ONPG) as substrate, β-galactosidase activity was measured by a modification of the method described by LEDERBERG[2]. 1 ml of the extract diluted to contain about 1000 μg of protein was added to 1 ml of 0.3 *M* sodium acetate buffer, pH 3.0, and 1 ml of 0.003 *M* ONPG. The incubation temperature was 35°. The reaction was terminated by the addition of 2 ml of M-K_2CO_3 and the *o*-nitrophenol released measured in a Klett-Summerson colorimeter using a 420 mμ (blue) filter. The hydrolysis of substrate at the final concentration (0.001 *M*) was shown to be linear with time during the assay period. When lactose was used as the substrate, the glucose released was measured manometrically according to the glucose oxidase method of KEILIN AND HARTREE[3]. Protein was determined by the method of LOWRY, ROSEBROUGH, FARR AND RANDALL[4] using Armour's bovine plasma fraction V as a reference standard.

Specific β-galactosidase activity is defined as the number of μmoles of substrate (ONPG or lactose) hydrolysed/1000 μg of protein/min.

RESULTS

pH optimum of β-galactosidase activity

The pH optimum of the β-galactosidase in crude extracts from strain L-cells and from the following organs was determined: adult brain, heart, intestine, kidney, liver, skeletal muscle, pancreas, and stomach; embryonic small intestine, liver, and muscle.

Curve 1 in Fig. 1 shows the β-galactosidase activity of the crude embryonic intestinal extract as a function of the pH. The enzyme is most active at pH 2.8. Curves obtained by plotting the β-galactosidase activities of crude extracts obtained from the other organs can be super-imposed on Curve 1. Curve 2 shows the activity of the embryonic intestinal enzyme after 22-fold purification. The pH optimum of this preparation is 2.2.

The β-galactosidase of crude extracts was irreversibly inactivated at pH values below 2.8. However, β-galactosidase was not inactivated by exposure to alkaline pH; after 1 h of incubation at pH 7.8, full activity could be recovered by re-adjustment of the pH to 2.8.

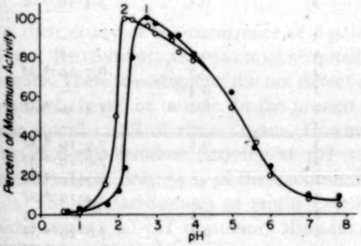

Fig. 1. Dependence of β-galactosidase activity on pH. ONPG was used as substrate. Sodium acetate buffer was used for the pH range 2.8 to 6.0, and Sørensen's phosphate buffer was used in the pH range 5.6 to 7.8. The final concn. of both buffers was 0.1 *M*. Curve 1 represents the β-galactosidase activity of crude embryonic intestinal extract, and Curve 2 represents the β-galactosidase activity of the embryonic intestinal extract purified 22-fold.

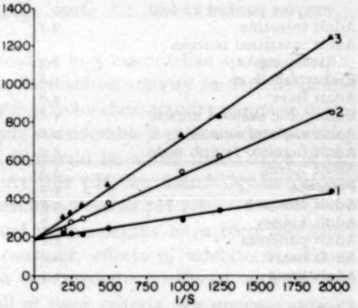

Fig. 2. Competitive inhibition by galactose and lactose of ONPG hydrolysis by strain L-cell and embryonic intestinal extracts. Curve 1, no inhibitor; curve 2, $5 \cdot 10^{-2}$ *M* lactose; curve 3, $1 \cdot 10^{-2}$ *M* galactose. The K_i values were calculated according to the formula $K_i = (I)/ mV/K_m - 1$. K_i for galactose, $3.4 \cdot 10^{-3}$ *M*; K_i for lactose, $2.6 \cdot 10^{-2}$ *M*.

MICHAELIS *constant of mouse β-galactosidase*

The MICHAELIS constants (K_m) were determined for strain L-cells and mouse organ β-galactosidase using ONPG as substrate. The results are summarized in Fig. 2 and column 5 of Table I. The values range from $5.4 \cdot 10^{-4}$ *M* for adult heart and intestine to $8.5 \cdot 10^{-4}$ *M* for adult brain and are probably within the range of experimental error.

MONOD, COHEN-BAZIRE AND COHN[5] have shown that the hydrolysis of ONPG by *Escherichia coli* β-galactosidase is inhibited by other galactosides in a competitive manner. Fig. 2 shows that the hydrolysis of ONPG by mouse β-galactosidase is inhibited in a competitive manner by galactose and lactose. The inhibition constant (K_i) for galactose is $3.4 \cdot 10^{-3}$ *M* and for lactose, $2.6 \cdot 10^{-2}$ *M*. At 10^{-1} *M* concentration, glucose and the α-galactoside, melibiose, do not inhibit mouse β-galactosidase.

It might also be stated here that purified intestinal β-galactosidase from suckling mice does not hydrolyse *o*-nitrophenyl-β-D-glucoside.

TABLE I

SPECIFIC ACTIVITIES AND MICHAELIS CONSTANTS OF MOUSE β-GALACTOSIDASE

Preparations of organ, tumor, and strain L-cell extracts and assays of specific β-galactosidase activity on ONPG were carried out as described under "MATERIALS AND METHODS". The determination of the MICHAELIS constants were carried out in pH 3.0 acetate buffer (0.1 *M*) using ONPG as substrate.

Source of enzyme	*Specific activity* $\times 10^3$	*No. of determinations*	*Standard deviation*	MICHAELIS *constant ONPG*
Embryonic intestine	52	3	5.5	$7.5 \cdot 10^{-4}$ *M*
Suckling intestine	46	2	3.9	—
Suckling intestine, enzyme purified 22-fold	1000	1	—	$7.6 \cdot 10^{-4}$ *M*
Adult intestine	2.1	3	0.1	$5.4 \cdot 10^{-4}$ *M*
Adult intestinal mucosa (scrapings)	1.3	1	—	—
Embryonic liver	1.8	2	0.2	—
Adult liver	2.8	4	0.7	$6.7 \cdot 10^{-4}$ *M*
Embryonic skeletal muscle	1.8	2	0.4	—
Adult skeletal muscle	0.6	2	0.2	$5.4 \cdot 10^{-4}$ *M*
Adult inguinal lymph node	5.7	1	—	—
Adult spleen	5.5	3	0.1	$7.9 \cdot 10^{-4}$ *M*
Placenta	4.2	2	—	$7.8 \cdot 10^{-4}$ *M*
Adult stomach	3.8	1	—	$6.7 \cdot 10^{-4}$ *M*
Adult kidney	3.7	1	—	$7.4 \cdot 10^{-4}$ *M*
Adult pancreas	2.2	2	0.2	—
Adult heart	0.6	2	0.2	$5.6 \cdot 10^{-4}$ *M*
Adult brain	0.4	2	0.1	$8.5 \cdot 10^{-4}$ *M*
Adult mammary gland tumor	6.4	2	0.5	—
Strain L-cells	2.7	6	0.3	$7.6 \cdot 10^{-4}$ *M*
Mouse Sarcoma I ascites tumor cells	2.3	2	0.1	—

Specific β-galactosidase activities

Table I shows that β-galactosidase activity was present in strain L-cells and all the mouse organs examined. It can also be seen that the organs differ widely with respect to their specific β-galactosidase activities. No β-galactosidase activity could be detected in mouse plasma, even on prolonged incubation with the substrate.

Specific β-galactosidase activity with lactose as substrate

Preparations of purified β-galactosidase from the intestines of suckling mice and strain L-cells were tested for their ability to hydrolyse lactose. The enzyme preparations were incubated at 35° with 0.1 *M* lactose at pH 3.0. The reaction was stopped by boiling the incubation mixture for 3 min and the glucose released was determined manometrically. Lactose was hydrolysed by both preparations at approx. one-third the rate at which they hydrolysed ONPG.

Precipitation of mouse β-galactosidase by specific antibody

Antiserum was prepared by injecting a rabbit with a purified β-galactosidase preparation (Table I) in Freund's adjuvant. A total of 4200 μg of purified extract protein was injected subcutaneously at weekly intervals over a 3-week period. One week after the final injection the rabbit was bled and the serum tested for anti-β-galactosidase activity by the method of COHN AND TORRIANI[6]. These authors found that specific antiserum precipitated, but did not inactivate *E. coli* β-galactosidase. Similarly, the β-galactosidase in crude extracts of embryonic mouse intestine, adult mouse liver, brain, muscle and strain L-cells was also precipitated but not inactivated by specific antiserum. Precipitin titers were too low to permit precise quantitative comparisons of the effectiveness of the antiserum in precipitating the β-galactosidase of the different organ extracts.

DISCUSSION

In their study of the occurrence of β-galactosidase in 5 mammalian species COHEN, TSOU, RUTENBURG AND SELIGMAN[7] noted β-galactosidase activity in various mouse organs. These investigators did not detect any β-galactosidase activity in mouse brain, stomach, heart, or muscle. In the present study considerable β-galactosidase activity was found in all of these organs. However, it should be noted that COHEN *et al.*[7] tested β-galactosidase activity at pH 5.0. At this pH, we found β-galactosidase activity to be only 55 % of the maximum activity shown at pH 2.8.

The β-galactosidases of strain L-cells and mouse organs have been compared with respect to pH optimum, MICHAELIS constant, effects of inhibitors, ratio of ONPG/lactose hydrolysed, and precipitation by antiserum produced with purified intestinal β-galactosidase. On the basis of all of these criteria, the enzyme appears to be the same molecular species, whatever its source.

Mouse strain L-cells have been propagated *in vitro* for many years. Thus, the persistence of the ability of these cells to form β-galactosidase is in contrast with the recent findings by LIEBERMAN AND OVE[8] and PERSKE, PARKS AND WALKER[9]. These authors have reported a great diminution or complete loss of several enzymes in tissue culture cells, compared with the organs of origin. It has also been suggested that neoplastic change may be accompanied by the loss of enzymes or enzyme function. It is of some interest to note that β-galactosidase activity was found in mouse Sarcoma I ascites tumor cells and at a relatively high concentration in spontaneous mammary gland tumors.

The observation that *all* mouse organ extracts contained appreciable amounts of β-galactosidase is surprising. Many of the organs, *e.g.*, spleen, lymph nodes, etc., showing rather high activities, would seem to have no functional requirement for the enzyme.

At least 2 hypotheses may be considered in order to explain these findings: (a) The β-galactosidase activity which was found in the different organs is associated with one cell type occurring in all organs, *e.g.*, macrophages. In this event differences in the specific β-galactosidase activity could reflect the relative abundance of this cell type in the various organs. (b) β-Galactosidase is present in all cells of the organs examined. If this were true, differences in specific activity among the individual organs and cell types could be interpreted as reflecting stages in biochemical differentiation.

The specific β-galactosidase activity of pure cultures of strain L-cells (fibroblasts) is lower than the specific β-galactosidase activity of the small intestine which is composed of many cell types including fibroblasts. This consideration would seem to exclude hypothesis (a), that β-galactosidase activity is associated with a single type of cell.

Little is known about mechanisms of biochemical differentiation. The finding that β-galactosidase occurs at a relatively high concentration in suckling mouse intestine—the organ in which the enzyme is functional—suggests that enzyme substrates may play a role in bringing about biochemical differentiation. Perhaps the high β-galactosidase activity of embryonic intestine may be related to the occurrence of lactose in the maternal circulation during pregnancy.

In order to test the possibility that some mammalian enzymes may be induced by their substrates, we have grown strain L-cells in the presence of galactosides. To date attempts to elicit increased β-galactosidase formation by this procedure have failed.

Finally, the observation that all mouse organs examined possess β-galactosidase activity suggests that every organ and possibly every cell of the organism may possess, to one degree or another, the enzymic potential of every other cell. Differences which do exist among the diverse cell types of the organism may be largely quantitative: the differentiated cell may possess in optimal amounts or in active forms those enzymes which are required for its specialized metabolic function.

ACKNOWLEDGEMENTS

This work was supported in part by State of Washington funds for medical and biological research and in part by a National Science Foundation research grant.

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A COMPARISON OF ION SHIFTS WITH RESPIRATION AND GLYCOLYSIS IN MUSCLE

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(Received March 17th, 1959)

SUMMARY

Changes in respiration and glycolysis have been correlated with ion shifts in toad muscle.

The effect of varying external levels of K^+, Rb^+, Cs^+ and Li^+ has been determined. High levels of K^+ increase the oxygen consumption of the tissue, and this effect passes through a maximum at approximately 18 mequiv./l K^+. The lactic acid production of muscle was significantly increased at external K^+ levels of 40 mequiv./l or greater. Rb^+ resembled K^+ in its effect on respiration, but did not significantly alter lactic acid production.

Cs^+ increased both respiration and glycolysis, but the effect did not show the peak response found with K^+ and Rb^+.

Li^+ caused an initial decrease in the rate of oxygen usage, and a later increase in this rate. These changes were associated with a decreased lactic acid production.

It is suggested that the increase in oxygen consumption in high external levels of the alkali metal ions is associated with an uncoupling of oxidative phosphorylation. This is likely to be a surface effect, and may be related to the depolarisation of the membrane.

INTRODUCTION

A series of experiments has been carried out in this laboratory to determine the effect of changes in the external ionic environment on the metabolism of muscle. The effect of these changes on the adenosine triphosphate and creatine phosphate levels has already been reported[1], and the results of a companion study on changes in respiration and glycolysis brought about by ion shifts will be reported in this communication.

Studies in this field have not been extensive[2,3]. Although the biochemistry of muscle is well understood, little work has been done to correlate this knowledge with the biophysics of the tissue. It would seem unlikely that one can dissociate the so-called physical characteristics of an excitable tissue from the metabolic function. Thus the relationship between the logarithm of the external K^+ level and the resting potential has been classed as a purely physical effect[4]. High levels of external K^+ are known, however, to bring about marked changes in the metabolism of the cell[1,2,5]. It is therefore desirable that the physical characteristics should be viewed in the light of the functional metabolism of the cell, and where possible changes in one should be correlated with changes in the other.

A fundamental difference of opinion exists in the literature concerning the nature of the ionic differentiation shown by the muscle cell[6-8]. It is generally believed that the accumulation of K^+ and partial exclusion of Na^+ results from the active transport of excluded constituents out of the cell. This may be linked with an active inward movement of accumulated ions. The energy requirements of such a system would be considerable, and if it could be shown that they exceed that obtainable from the resting metabolism this would constitute a refutation of the theory. The difficulties of making such an estimate have been discussed previously[1,9]. The results which are presented here, in conjunction with those reported in the preceding paper[1], suggest that such a channelling of metabolic energy for ion transport does not occur. It would seem, however, that on existing knowledge no definite answer can be given.

METHODS

The sartorius muscle of the toad, *Bufo marinus*, was used in this study. The treatment of muscles was similar to that reported previously[1]. One of a pair of muscles was always used as the control. The rate of oxygen uptake was measured by conventional Warburg techniques. Muscles weighing less than 150 mg were used to minimize the delay in the diffusion of oxygen to fibres in the interior of the muscle. Lactic acid was estimated by the method of BARKER AND SUMMERSON[10]. Both the lactic acid which diffused into the Ringer solution in the Warburg flask and the amount in the muscle was estimated, and added together to give the amount produced during the period of observation. It was found possible to carry out ionic analyses on the same muscle by cutting it in two at the end of the run, and using one half for the lactic acid estimation, and the other for the ionic analysis. It was shown previously[11] that the ionic content of the 2 halves of a muscle is identical.

The cation content of the muscles was determined using a Beckman flame spectrophotometer. Due care was taken to correct for the mutual interference between the ions, which was particularly great[1] in solutions containing mixtures of K^+ and Rb^+ or Cs^+.

The solutions used will be detailed in the text[12]. A mixture of antibiotics was added to the Ringer in all experiments of longer than 4 h duration, since slight bacterial contamination may give spuriously high figures for the oxygen consumption.

RESULTS

The effect of alteration of the external K^+ level

High external levels of K^+ are known to affect muscle in a number of ways. Firstly the so-called physical characteristics of the membrane are changed. The resting potential falls off, and above a level of approx. 10 mequiv./l it is possible to relate the decline in potential with the logarithm of the external K^+ concentration[13]. The membrane conductance is increased[14], and the flux rates of Na^+ and K^+ are raised[15,16].

The metabolism is altered, since it has been shown that the resting heat rate is markedly increased[2] in high K^+, as is also the oxygen uptake[5]. An increase in lactic acid formation was also reported[5]. We noted in the preceding paper[1] that K^+ tended to decrease the ATP and CrP level of the tissue.

The oxygen uptake of the muscles was followed over a 4-h period. The muscles were soaked in Ringers containing amounts of KCl varying from zero to 100 mequiv./l, in addition to the normal constituents. No alteration in the Na^+ content of the Ringer was made, as a reduction in the level of this ion in proportion to the increase in K^+ would have led to a swelling of the tissue, and would also have constituted another variable in the system. The companion muscle held in normal Ringer was used as the control.

At least 4 pairs of muscles were used at each level of K^+, and it was usual to repeat each experiment several times, as the response of different batches of toads tended to vary[12]. The lactic acid content of both the muscle and the immersing Ringer was determined at the end of the run, and the ionic content was also estimated as was stated in the METHODS section.

The results of these experiments are set out in Table I, and they have been depicted graphically in the trend curves shown in Fig. 1.

TABLE I

THE EFFECTS OF K^+ ON RESPIRATION, GLYCOLYSIS AND IONIC CONTENT

Only figures marked with an asterisk are significantly different from controls, $P < 0.05$. Ionic contents refer to total concentration uncorrected for the extracellular space.

K^+ level in Ringer (mequiv./l)	*Oxygen uptake µl/mg*		*Lactic acid mmoles/kg*		*Na mequiv./kg*		*K mequiv./kg*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
K-free	0.235	0.245	21.04	20.22	59.06	64.75	62.20	53.44*
7.5	0.197	0.205	15.19	14.80	64.73	65.88	83.48	81.50
10	0.187	0.247*	14.78	15.11	47.33	51.60	86.25	84.25
15	0.206	0.515*	16.13	17.97	57.99	59.94	78.51	85.76
21.5	0.245	0.530*	27.74	27.69	61.97	60.23	67.14	74.86*
30	0.223	0.360*	18.65	23.36	64.38	72.48	78.40	86.40
43.0	0.271	0.346*	21.47	25.87*	64.26	59.54*	58.66	70.91*
50	0.209	0.259	14.48	20.64*	66.00	68.55	83.55	116.23*
60	0.191	0.263*	15.14	20.60*	61.55	64.35	78.63	105.38*
100	0.219	0.319*	16,14	21.13*	71.03	64.43	80.40	159.00*

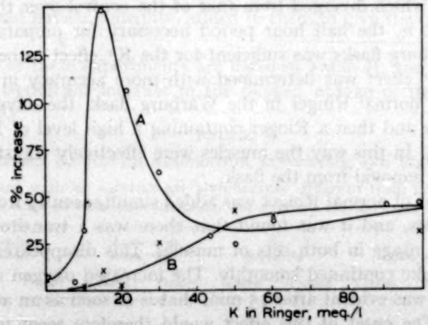

Fig. 1. The effect of alteration of the external K^+ level on the oxygen uptake and lactic acid production of muscle. Curve A is a trend curve illustrating the change in oxygen uptake. Curve B is a trend curve showing the change in lactic acid production.

K-free Ringer: No increase in oxygen uptake was found in muscles held in K-free Ringer compared to controls in normal Ringer, nor was there any change in lactic acid formation[5]. The K^+ content of the muscles in the K-free solution showed a small but statistically significant decrease compared with the controls, when they were both analysed at the end of the run. Moreover, both sets of muscles could have been expected to suffer the usual loss of K^+, and gain of Na^+ found on soaking in Ringer[6]. Consequently the Ringer in the Warburg flask would have contained some K^+ derived from the muscle loss, and it would be more accurate to say that *reduction* in the external K^+ level produced no metabolic change.

High KCl Ringer: Increase in the external K^+ level brought about a rise in oxygen consumption which was significant at all levels of K^+ equal to or greater than 10 mequiv./l. The increase in oxygen usage passed through a maximum at between 15 and 20 mequiv./l KCl. This peak was checked by comparing the oxygen uptake of companion muscles in 21.5 and 43 mequiv./l K^+. It was shown that the muscles in the lower K^+ solution showed an oxygen uptake which was over 100 % greater than was found in the more concentrated K^+ solution.

The production of lactic acid was also increased by high external K^+. The effect was only statistically significant at levels of K^+ equal to or greater than 43 mequiv./l, and appeared to increase with increase in the external K^+ level in a roughly linear fashion.

The high K Ringer brought about no consistent change in the Na^+ content of the tissue[6].

There was a significant increase in the total K^+ content of the tissue only at external levels of K^+ of 21.5 mequiv./l and above. The figures shown in Table I have not been corrected for the extracellular space contribution of this ion, but the increases in K^+ are of the same order of magnitude as those reported previously[6], and are smaller than would be expected from the DONNAN theory for K^+ accumulation.

Rate of onset of effect

The graph of oxygen consumption against time was linear both in the control muscles and in muscles in high K Ringer. Muscles in the latter medium showed an oxygen usage curve which diverged from that of the control over the entire period of observation. That is, the half hour period necessary for preparation and equilibration of the Warburg flasks was sufficient for the K^+ effect to be fully revealed. The onset of the K^+ effect was determined with more accuracy in muscles which were equilibrated in normal Ringer in the Warburg flask, the oxygen uptake was followed for 115 min and then a Ringer containing a high level of KCl was tipped in from the side arm. In this way the muscles were effectively transferred to a high K^+ solution without removal from the flask.

A similar amount of normal Ringer was added simultaneously from the side arm in the control muscles, and it was found that there was a transitory inflection in the curve of oxygen usage in both sets of muscles. This disappeared after 15 min, and the oxygen uptake continued smoothly. The increased oxygen consumption in the high KCl Ringer was evident after 15 min, that is as soon as an accurate reading could be obtained. The onset of this effect would therefore seem to be rapid (see Fig. 2).

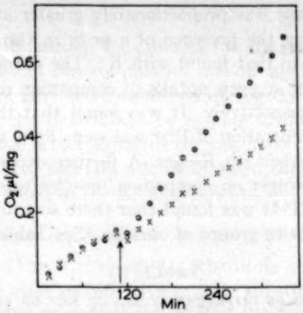

Fig. 2. The time of onset of the increased oxygen uptake of muscles in 21.5 mequiv./l KCl Ringer. Both groups of muscles were incubated in the normal Ringer until the point marked with the arrow. At this point equal vols. of high KCl Ringer or normal Ringer were tipped from the side arm into the flasks. The upper curve (circles) shows the response of the muscles to the high K^+ solution. Each point is the mean of ten observations.

The effect of high RbCl Ringer

Rb^+ is known to resemble K^+ in many ways in its action on the living cell[17]. The decrease in resting potential brought about by Rb^+ appears to be similar to, but less in extent, than the K^+ depolarisation[2]. HILL AND HOWARTH[2] found the increment in heat rate in Rb^+ solutions had a higher threshold than was found for K^+ and the effect was smaller in magnitude. It was noted previously from this laboratory that Rb Ringer brought about a decrease in the level of creatine phosphate, but not of adenosine triphosphate, at 20 mequiv./l RbCl. Thus the uncoupling of oxidative phosphorylation by Rb^+ would appear to be less effective than was found with K^+.

A series of experiments was carried out with Rb Ringer which was similar to, but less extensive than, that reported in the previous section for K^+. RbCl was added to normal Ringer, which already contained 2.7 mequiv./l KCl. Thus the levels listed in Table II, should have 2.7 mequiv. added to them to give the content of $K^+ + Rb^+$ in the Ringer.

The results of these experiments are listed in Table II, and it may be seen that RbCl caused a significant increase in the oxygen uptake of the tissue at all levels

TABLE II

THE EFFECT OF Rb^+ ON RESPIRATION, GLYCOLYSIS, AND IONIC CONTENT

Only figures marked with an asterisk are significantly different from controls, $P < 0.05$. Ionic contents refer to total concentration uncorrected for the extracellular space.

Rb level in Ringer mequiv./l	*O_2 uptake µl/mg*		*Lactic acid mmoles/kg*		*Na mequiv./kg*		*K mequiv./kg*		*Rb mequiv./kg*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
7.5	0.199	0.375*	19.01	20.35	65.83	62.23	66.8	49.2*		—
15	0.199	0.372*	9.70	11.10	57.8	47.8	75.1	57.6*		29.9
30	0.196	0.291*	26.84	23.87	87.1	76.5	71.8	35.7*		47.7

of Rb^+ tested. The increase was proportionately greater at 15 mequiv./l Rb^+ than at 30 mequiv./l, indicating the presence of a peak in the oxygen uptake curve at this level of Rb^+ similar to that found with K^+. The authenticity of this peak was checked by comparing the oxygen uptake of companion muscles in solutions of 15 and 30 mequiv./l RbCl respectively. It was found that the oxygen uptake of the muscles in the lower concentration of Rb^+ was some 85 % greater than that of companion muscles in the higher Rb Ringer. A further experiment was performed in which the effect of Rb Ringer on companion muscles was determined at levels of 30 and 60 mequiv./l RbCl. It was found that there was no significant difference in the oxygen uptake of the two groups of muscles. (See Table III.)

TABLE III

A COMPARISON OF THE EFFECT OF DIFFERENT LEVELS OF Rb^+ ON RESPIRATION AND GLYCOLYSIS

Only figures marked with an asterisk are significantly different from controls, $P < 0.05$. Ionic contents refer to total concentration uncorrected for the extracellular space.

Rb^+ in Ringer, mequiv./l	15	30	30	60
Oxygen uptake, µl/mg	0.507	0.273*	0.224	0.262
Lactic acid, mmoles/kg	16.7	13.4	19.4	17.7
Na, mequiv./kg	70.5	66.9	61.5	58.6
K, mequiv./kg	51.8	35.5*	39.4	34.8
Rb, mequiv./kg	38.5	55.7*	39.7	66.8*

These results resemble closely those obtained for K^+ in the position of the maximum in the curve of oxygen usage versus external concentration of Rb^+. This finding is at variance with that of HILL AND HOWARTH[2], who found a higher threshold for the response of the heat rate to Rb^+ than to K^+. These authors also noted that the response to Rb^+ was less in extent than the K^+ response. We found that the average increase in oxygen usage of toad muscles in 15 mequiv./l KCl was 150 %, whereas the average increase in a similar concentration of RbCl was only 87 %. It appeared suggestive that Rb^+ was less effective than K^+ in increasing the oxygen uptake, but data using companion muscles showed that this was not so (see below).

The production of lactic acid was not significantly increased in the Rb^+ treated muscles compared to controls in normal Ringer. At 15 mequiv./l external Rb^+, there was an appreciable increase in the lactic acid production, but this was not significant at the 5 % level. When the effect of 15 mequiv./l RbCl was compared with companion muscles in 30 mequiv./l RbCl there was a similar non-significant increase in lactic acid formation at the lower Rb^+ level. There was no change in the lactic acid formation at 30 or 60 mequiv./l RbCl. This ion therefore appears to differ from K^+ in its effect on glycolysis.

Rb Ringer brought about no alteration in the Na^+ content of the cell at any Rb^+ level tested.

There was a significant decrease in the K^+ content of the cell at 15 and 30 mequiv./l RbCl. The K^+ loss was made up by an entry of Rb^+ into the cell, and this ion was accumulated above the external level in all experiments[1,18].

Rate of onset of effect

As was noted in the K^+ section the difference in the graph of oxygen usage with time between the treated and control muscles was apparent virtually from the first reading. Thus the effect of Rb^+ was established during the equilibration period. The exact time sequence was not determined by tipping in the RbCl solution from the side arm, as was done in the K^+ experiments. It was, however, noted that the increase in oxygen uptake in the Rb^+ solution was maintained throughout the 4-h period of observation, as was also found in the K^+ experiments.

The effect of high CsCl Ringer

It is generally agreed that Cs^+ resembles Rb^+ in its ability to displace K^+ from the cell, though it is somewhat less effective as a K^+ substitute[1,18] than is the smaller Rb^+. It was reported in the preceding paper[1] that Cs^+ brings about changes in the level of creatine phosphate in the muscle which follow a different pattern to those found with high external levels of K^+ and Rb^+. These latter named ions reduced the high energy phosphate content only at levels of 20 mequiv./l, whereas Cs^+ brought about decreases in creatine phosphate at all the levels tested.

A series of experiments was carried out with high CsCl Ringer which was similar to that reported for K^+ and Rb^+. Since the entry of Cs^+ into muscle is slower[18] than is found for Rb^+, some of the experiments were carried out for longer than the usual 4 h. The results of these experiments are set out in Table IV.

There was a significant increase in the oxygen uptake of the muscles at levels of external Cs^+ of 30 mequiv./l and above. We were unable to demonstrate a peak in the curve of oxygen usage against external Cs^+ level such as was found in the K^+ and Rb^+ series. The failure of the response to pass through a maximum was checked in experiments in which the oxygen uptake of companion muscles was compared in solutions containing 15 or 30 mequiv./l CsCl; and 30 or 60 mequiv./l CsCl respectively (see Table V). There was no significant difference between the oxygen uptake of paired muscles in either series.

TABLE IV

THE EFFECT OF Cs^+ ON RESPIRATION, GLYCOLYSIS AND IONIC LEVELS

Only figures marked with an asterisk are significantly different from controls, $P < 0.05$. Ionic contents refer to total concentration uncorrected for the extracellular space.

Cs^+ level in Ringer	*O_2 uptake µl/mg*		*Lactic acid mmoles/kg*		*Na mequiv./kg*		*K mequiv./kg*		*Cs mequiv./kg*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
7.5	0.180	0.200	8.23	8.60	87.5	93.5	78.0	63.4	—	6.2
15	0.180	0.219	11.52	12.72	26.1	27.2	113.7	114.4	—	7.9
30	0.160	0.275*	20.40	31.78*	60.4	71.5*	75.3	53.4*	—	18.1
100	0.195	0.357*	18.18	21.57*	58.3	70.9*	81.3	56.0*	—	24.5
(8 h expt.)										
30	0.364	0.691*	13.62	17.64*	60.2	53.8*	70.6	41.5*	—	20.7
(16 h equilibration)										
30	0.220	0.415*	15.73	14.06	82.0	80.2	80.4	34.5*	—	52.1

TABLE V

A COMPARISON OF THE EFFECT OF DIFFERENT LEVELS OF CsCl

Only figures marked with an asterisk are significantly different from controls, $P < 0.05$. Ionic contents refer to total concentration uncorrected for the extracellular space.

Cs^+ in Ringer, mequiv./l	15	30	30	60
O_2 uptake, µl/mg	0.258	0.291	0.277	0.286
Lactic acid, mmoles/kg	11.9	12.8	8.4	7.8
Na, mequiv./l	54.8	75.2*	91.3	95.3
K, mequiv./l	64.6	53.0	67.1	58.7
Cs, mequiv./l	10.2	20.4*	19.7	37.2*

Two experiments were carried out of 8 and 20 h duration. In the 8-h experiment the oxygen uptake was followed for this length of time, and consequently the figure listed in Table IV refers to the cumulative oxygen uptake. In the 20-h experiment the muscles were agitated in the Warburg flasks for 16 h, then the oxygen uptake was followed for 4 h. The average increase in the oxygen uptake in Cs Ringer during the first 4 h of immersion was found to be 72 %. The average increase in the oxygen uptake in a 4-h period subsequent to 16-h equilibration in the Cs^+ solution was 89 %. It would seem that the increment is too small to be considered an effect of the increased period of incubation.

The lactic acid production of the tissue was increased in Ringers containing 30 or 100 mequiv./l CsCl. Like the oxygen uptake this increase did not pass through a maximum, but was roughly similar in magnitude at all levels (see Tables IV and V).

When the production of lactic acid was compared in 30 mequiv./l CsCl at 4, 8 and 20 h, it was found that the increment became less with increasing time. Thus at 4 h there was an increased lactic acid production over the control level of 56 %. After 8 h this figure had fallen to 29 %, while after 20 h there was a non-significant decrease of 11 %.

The Cs Ringer showed a tendency to increase the Na^+ content of the muscle, but this was not a regular or consistent finding.

There was a reduction in the K^+ content of the muscles which was significant at 30 and 100 mequiv./l Cs^+ and which increased with increased time of soaking in the Cs^+ solution. Cs^+ entered the muscles more or less in proportion to the K^+ loss when average values were compared. When, however, a correlation in individual muscles was attempted the Cs^+ and K^+ movements could not be considered reciprocal.

It should be noted that the increased Cs^+ content of the tissue with increased time of soaking did not result in a proportionate increase in the oxygen uptake, and consequently the increased respiration in Cs Ringer cannot be related to the intracellular level of this ion.

Rate of onset of effect

An inspection of the graph of oxygen consumption against time showed, as was found in the K^+ and Rb^+ sections, that the increase in the oxygen uptake in the Cs Ringer was apparent virtually from the first reading. There was some suggestion of a delay in the onset of the increase in oxygen consumption in perhaps 30 % of the muscles, which persisted for upwards of 60 min. The effect was not marked, and could be considered to be almost within the error of the method. It is of interest that the increased oxygen uptake was sustained for 20 h at about the same rate as was established during the first 4 h in the test Ringer.

A correlation of the effect of K^+, Rb^+ and Cs^+

Since the response of the muscle to the test Ringer may vary markedly in degree from one toad to another, it is not possible to say from average figures whether one ion is more effective than another in increasing the respiration. Consequently experiments were carried out in which companion muscles were immersed in K^+ or Rb^+ solutions, in K^+ or Cs^+ solutions, or in Rb^+ or Cs^+ solutions. The comparisons were carried out at levels of 20 or 40 mequiv./l in each instance. The results of these experiments are set out in Table VI.

TABLE VI

A COMPARISON OF THE EFFECT OF K, Rb AND Cs ON RESPIRATION AND GLYCOLYSIS

The "control" muscle is that muscle which is immersed in the first mentioned solution in each experiment. Thus in the K *vs.* Rb experiment the K treated muscle is considered the control. Only figures marked with an asterisk are significantly different from controls, $P < 0.05$. Ionic contents refer to total concentration uncorrected for the extracellular space.

Concentration in Ringer, mequiv./l	*K vs. Rb*		*K vs. Cs*		*Rb vs. Cs*	
	20	*40*	*20*	*40*	*20*	*40*
Oxygen uptake, µl/mg						
Control	0.558	0.259	0.611	0.298	0.689	0.296
Treated	0.552	0.227	0.313*	0.368	0.364*	0.341
Lactic acid, mmoles/kg						
Control	18.1	17.3	18.3	16.0	21.6	19.1
Treated	19.2	17.1	15.6	16.9	19.9	20.4
Na, mequiv./kg						
Control	86.1	76.8	52.9	42.4	55.9	53.8
Treated	89.7	79.9	46.3	51.4	77.6*	64.9
K, mequiv./kg						
Control	58.4	106	87.0	96.2	34.2	27.6
Treated	22.9*	24.8*	75.5	51.6*	48.4*	58.4*
Rb, mequiv./kg						
Control	—	—			48.1	69.5
Treated	30.3	77.0			—	—
Cs, mequiv./kg						
Control			—	—	—	—
Treated			13.2	12.0	13.0	12.0

It may be seen that there was no significant difference between the oxygen uptake of muscles in the K^+ or Rb^+ solution at either level tested. As was to be expected the oxygen uptake of both sets of muscles was greater at the 20-mequiv./l level than at the 40-mequiv./l level. When however K^+ was compared with Cs^+ it was found that the respiration of the K^+ treated muscles was significantly greater than the Cs^+ muscles at 20 mequiv./l, whilst the Cs^+ treated muscles showed an insignificant increase in oxygen uptake over the K^+ muscles at 40 mequiv./l. The comparison of Rb^+ with Cs^+ confirmed the similarity between the action of K^+ and Rb^+. Here again the Rb^+ treated muscles showed a significant increase in oxygen uptake compared to the Cs^+ muscles at 20 mequiv./l and there was a statistically insignificant reversal of the effect at 40 mequiv./l.

There was no significant difference in the production of lactic acid between companion muscles in any of the experiments.

The ionic results conform to the pattern found in the previous tables. It is of interest that Rb^+ is more effective than Cs^+ in displacing cellular K^+ at both levels compared.

Thus the difference in heat rate reported by HILL AND HOWARTH[2] between K^+ and Rb^+ solutions is not reflected in the toad by a difference in respiration.

The effect of LiCl Ringer

Li^+ differs from Rb^+ and Cs^+ in that it may be regarded as a partial substitute[7] for cellular Na^+, whereas Rb^+ and Cs^+ appear to replace cellular K^+. The only interaction between Li^+ and the normal ionic constituents of the cell was found[7] to take place with Na^+. Experiments on the rate of efflux of ^{22}Na from the cell showed that Li^+ treatment increased the amount of Na^+ in the slowest moving fraction of the efflux.

It was reported previously that substitution of half the Ringer Na^+ with Li^+ did not alter the level of high energy phosphate compounds in the muscle, even over an 18-h soaking period. A companion study has now been carried out to determine the effect of Li^+ on respiration and glycolysis. Since the rate of Li^+ efflux from the cell is markedly diminished in muscles which are equilibrated in the Li^+ solution for upwards of 18 h the metabolic experiments were carried out for 4 or 20-h periods. The test Ringer had half the Na^+ (65 mequiv./l) replaced with Li^+. Companion muscles held in normal Ringer were used as controls, in the usual way. The results of these experiments are set out in Table VII.

TABLE VII

THE EFFECT OF Li^+ ON RESPIRATION, GLYCOLYSIS AND IONIC CONTENT

The test Ringer contained 65 mequiv./l Na^+ and 65 mequiv./l Li^+. In the 20-h experiment the muscles were equilibrated in the Warburg for 16 h, then the oxygen uptake was followed for 4 h. Only figures marked with an asterisk are significantly different from controls, $P < 0.05$. Ionic contents refer to total concentration uncorrected for the extracellular space.

		4 h	*20 h*
O_2 uptake, µl/mg	Control	0.215	0.156
	Treated	0.158*	0.239*
Lactic acid, mmoles/kg	Control	20.94	19.71
	Treated	17.28*	15.18
Na, mequiv./kg	Control	67.4	61.2
	Treated	33.8*	27.9*
K, mequiv./kg	Control	72.9	72.6
	Treated	66.4	60.5
Li, mequiv./kg	Control	—	—
	Treated	25.8	41.4

It may be seen that the oxygen uptake of muscles in the Li Ringer was significantly less than that of the controls at the shorter time of soaking. However, in those muscles which were equilibrated for 16 h in the Li^+ solution before the 4-h recording of the oxygen uptake this effect was reversed, and the oxygen uptake was significantly greater in the test Ringer.

The lactic acid production was less in the treated muscles at both times of observation, but the decrease was only statistically significant in the 4-h experiment.

The results of the ionic analyses are essentially similar to those reported previously. The Na^+ content of the tissue fell as the external level of this ion was lowered, and Li^+ entered the cell to replace it. The entry of Li^+ into the cell was greater at the longer time of soaking. The sum of $Li^+ + Na^+$ was slightly less than the level of Na^+ in the control muscle at 4 h, but was rather greater than this level after 20 h.

The treated muscles showed slight, and statistically insignificant losses of K^+, compared to controls in normal Ringer. This loss increased with time.

The difference in the pattern of the ionic results at the two times of soaking would not seem great enough to account for the reversal of the effect of Li^+ on the oxygen uptake, nor could this effect be correlated with the findings that the adenosine triphosphate and creatine phosphate levels of the cell were not altered by Li^+.

DISCUSSION

In discussing the energy requirements for the active transport of ions into and out of the cell[1,3,9], little consideration has been given to the question of what proportion of the energy derived from respiration can be effectively channelled for work, and what proportion is dissipated as heat. There is a growing realisation[1,2,4] that increases in the oxygen uptake of resting tissue may not be related to increases in the work potential of the tissue, but may result from the uncoupling of oxidation from the formation of high energy phosphate bond compounds. It is known that unfavourable conditions depress phosphorylation in isolated mitochondria while active respiration is still proceeding[19]. It would seem that the increases in oxygen uptake in muscles in high levels of K^+, Rb^+ and Cs^+ coincide with a similar uncoupling.

The increases in heat rate reported by HILL AND HOWARTH[2] for frog muscle occur at the same K^+ levels as we noted to affect the oxygen uptake of toad muscle. The effect of K^+ on the oxygen uptake showed a maximum response some 150 % greater than the control, whilst the increase in heat rate could be as much as 20 to 30 times the basal level. HEGNAUER *et al.*[5] reported proportionately greater increases in oxygen uptake in frog than we were able to demonstrate, and it may be that this animal is more sensitive to high K^+ levels than is the toad. Whether or not this is so, the additional heat production must be considered as an uncoupling of oxidation from energy consuming processes, and must be due to a decreased efficiency of the system.

It is generally assumed that the energy for synthetic processes, muscular contraction, and secretion is almost exclusively obtained from the fission of ATP. Consequently the energy for ion transport would seem, in the final analysis, to derive from ATP[20]. In their estimate of the energy requirements of the Na^+ pump KEYNES AND MAISEL[3] have been concerned merely with the relation between oxygen uptake and Na^+ extrusion. If the increase in oxygen uptake in raised levels of external K^+ cannot be regarded as an increase in the functional activity of the tissue, then one must query their conclusion that the increased rate of Na$^+$ extrusion is correlated with the increased metabolic rate.

Further, these authors noted that the rate of oxygen uptake tended to rise with time, and in lengthy experiments the final rate was sometimes well above the initial rate. HEGNAUER *et al.*[5] found that the rate tended to fall off with time, and we were able to show that the increased rate of oxygen uptake showed a remarkable constancy for upwards of 20 h. The heat rate[2] was shown to undergo either an upward or downward "creep" after the muscle was removed from the test solution, but this may have been related to the fact that measurements were carried out in the gaseous phase. Since KEYNES AND MAISEL[3] do not report the use of antibiotics in their experiments it may well be that their Warburg flasks were bacterially contaminated. We have found it impossible, even using sterile surgical techniques to obtain bacteria free muscles. In experiments of more than 4-h duration in which antibiotics were not used it was possible to demonstrate an exponential rise in the rate of oxygen uptake (DE LA LANDE, private communication).

HEGNAUER *et al.*[5] have suggested that both the break down of creatine phosphate, and the increase in the production of lactic acid in high K Ringer may act as pacemakers in setting the rate of oxygen consumption. Although we have confirmed that these are concomitant phenomena, it is difficult to envisage a cause and effect relationship. The cause would seem to lie in the uncoupling of oxidative phosphorylations, but we are not able to see how this is brought about by the test solutions.

HILL AND HOWARTH[2] suppose that the metabolic disturbance in high K$^+$ solutions can be related to the dis-orientation of ordered monolayers, which might be transmitted from the surface inwards. This disordering could conceivably disturb some regulatory system within the cell.

This still leaves us with the problem of why the response to high external K$^+$ levels passes through a maximum at about 18 mequiv./l K$^+$ (or Rb$^+$). One could suppose that the constraint of surface polarisation on the orientated monolayers would decrease with increasing depolarisation, unless a partial disorientation is presumed to be more effective than a complete disorientation. The only clue resides in the fact that the maximal response occurs at a level of the resting potential which coincides with the threshold. It may be that lowering the potential to this level alters the physical chemistry of the surface in such a way as to set in train a series of events, which include the firing of the impulse and the potentiation of the rate of oxygen uptake.

In this respect the work of HILL *et al.*[21] on the heat production accompanying a nervous impulse is of interest. These authors noted a heat production which was associated with the active phase of the impulse, and a slower heat absorption which persisted for some 300 msec after the impulse. One wonders whether the partial depolarisation of the membrane with KCl is related to that found during the initiation of the impulse, but in the case of the KCl depolarisation the system is "jammed" at this point.

It is known that both the heat rate[2] and the threshold[22] are sensitive to Ca^{++}–Mg^{++} interactions. These ions also alter the sol–gel relations of cytoplasm[23]. Whether these effects can be correlated with the above hypothesis must await further work.

The similarity of the metabolic effects produced by K$^+$ and Rb$^+$ has been noted previously[1]. Cs^+ does not fit into the pattern of results obtained with these ions, and Li^+ (as was to be expected) has an entirely different action. The metabolic response to Li^+ will be considered elsewhere when discussing our results on the effect of high Na^+ solutions on respiration and glycolysis.

It is of interest to consider whether the increase in the rate of oxygen uptake can be considered a surface phenomenon, as would be expected from the above discussion. It was noted in the results section that the rate of onset of the effect in the case of K^+ and Rb^+ at least was very rapid. This would indicate that the response is associated either with the membrane or the surface layers of the cell. It will be shown elsewhere[18] that Rb^+ and Cs^+ cross the membrane with considerable facility, but they displace the cellular K^+ at an appreciably slower rate. The suggestion of a surface effect is further strengthened, however, by the fact that we were unable to correlate the increase in oxygen uptake with the cellular level of Rb^+ or Cs^+. HILL AND HOWARTH[2] were also able to demonstrate a rise in heat rate under conditions which either raised or lowered the intracellular K^+ level.

ACKNOWLEDGEMENTS

This work was carried out with the aid of a grant from the National Health and Medical Research Council of Australia. Mrs. S. E. SIMON was the recipient of a Burroughs-Wellcome (Australia) Fellowship. The authors wish to express their thanks to Professor F. H. SHAW for his help and encouragement.

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L'ALDOSE-RÉDUCTASE

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(Reçu le 1 avril 1959)

SUMMARY

Aldose reductase

The seminal vesicles and placenta of sheep contain an enzymic system, called aldose reductase, that catalyses the reduction by TPNH of a great number of aldoses or related substances, and of several aliphatic and cyclic aldehydes. Notably glucose is reduced to sorbitol and glucosone to fructose. Rat liver contains an analogous enzymic system, but this is inactive towards glucose and certain pentoses, and the name "aldehyde reductase" is suggested for it.

INTRODUCTION

Ainsi que nous le décrivons dans la publication suivante[1], les vésicules séminales de mouton transforment le glucose en sorbitol. Cette transformation se fait sous l'action d'un système enzymatique qui catalyse la réduction de nombreux aldoses en polyalcools par le TPNH**, selon l'équation générale:

$$RCHO + TPNH + H^+ \rightleftharpoons RCH_2OH + TPN^+$$

Plusieurs aldéhydes, n'appartenant pas au groupe des oses, réagissent dans le même système enzymatique qui n'a pas été suffisamment purifié pour que sa spécificité soit connue avec certitude. Nous lui avons donné le nom d'"aldose-réductase" par analogie avec celui de "cétose-réductase" que WILLIAMS-ASHMAN ET BANKS[2] ont donné à la sorbitol-déshydrogénase[3] qui catalyse la réduction réversible de divers cétoses par le DPNH.

Le nouvel enzyme a également été trouvé dans le placenta de brebis et de vache mais non dans celui de la lapine. Le foie, le cerveau et le rein de divers animaux contiennent un système enzymatique analogue mais de spécificité plus restreinte que l'enzyme placentaire ou vésiculaire.

Les observations que nous décrivons dans cette publication et dans celle qui la suit ont fait l'objet d'une note préliminaire[4] et ont été partiellement décrites dans une monographie[5].

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* Associé du F.N.R.S.

** Nous utilisons les abréviations suivantes: DPN pour diphosphopyridine-nucléotide. TPN pour triphosphopyridine-nucléotide, EDTA pour éthylènediaminetétraacétate.

MÉTHODES

Les organes provenant de moutons ou de vaches sont obtenus fraîchement de l'abattoir. Ils sont broyés au Waring-Blendor dans deux volumes d'eau froide. Un extrait clair est obtenu en écartant le matériel qui sédimente au cours d'une centrifugation de 1,500,000 $g \times$ min. Ces extraits sont éventuellement dialysés en chambre froide contre un tampon phosphate 0.003 M à pH 7.4 ou encore incubés pendant 15 min à 18° en présence d'EDTA 0.01 M; ainsi que nous le décrivons dans la publication suivante[1], ce dernier traitement a pour effet d'inactiver complètement la cétose-réductase.

La glucosone a été préparée par action de l'acide pyruvique sur la glucosazone[6,7], la D-glycéraldéhyde par la méthode de BAER ET FISCHER[8], les heptoses sont dus à la générosité du Dr. N. K. RICHTMYER. Nous avons utilisé du glucose Merck, de la DL-glycéraldéhyde et de la dioxyacétone Paragon, les autres sucres étant préparés par la firme Roche. Le [^{14}C]glucose a été fourni par le "Radiochemical Centre" d'Amersham, le TPN, le TPNH et le DPN par la firme Sigma; le TPNH a également été préparé par réduction du TPN$^+$ sous l'action de la glucose-6-phosphate-déshydrogénase ou parfois de l'isocitrico-déshydrogénase présentes dans l'extrait utilisé.

Le sorbitol est dosé photométriquement par la méthode de WEST ET RAPOPORT[9] comme modifiée par FAULKNER[10]. Les contributions du glucose et du fructose à ce dosage sont d'environ 2 et 15 % de celle du sorbitol, celle de l'inositol est nulle. Le glucose est mesuré spécifiquement au moyen de la glucose-oxidase suivant HUGGETT ET NIXON[11]. Les autres méthodes de dosage ont été décrites antérieurement[12].

Pour la séparation chromatographique des sucres, nous utilisons comme solvant soit le mélange butanol–éthanol–eau (52.5 : 32 : 15.5), soit le phénol saturé d'eau. Ce dernier solvant permet le plus souvent de séparer le sorbitol du fructose. L'extraction des tissus se fait par un mélange en quantités équimoléculaires de sulfate de zinc et d'hydroxyde de baryum, l'extrait étant ensuite désionisé par passage sur une colonne d'échangeurs d'anions (Amberlite IR 45) et de cations (Amberlite IR 120), puis concentré par lyophilisation.

RÉSULTATS

1. *Mise en évidence et spécificité de la réaction*

L'étude de l'aldose-réductase dans les extraits de tissus est facilitée par la présence de la glucose-6-phosphate-déshydrogénase qui fournit facilement du TPNH à partir de TPN$^+$ et de glucose-6-phosphate. En présence d'une quantité limitée d'hexoses-phosphates, on peut suivre spectrophotométriquement la réduction du coenzyme et sa réoxydation par le glucose ou d'autres aldoses (Fig. 1). Les deux réactions peuvent aussi progresser simultanément et dans ce cas une quantité catalytique de TPN suffit au couplage; on voit à la Fig. 2 qu'un extrait de vésicules séminales de mouton fait ainsi disparaître différents hexoses, pentoses et trioses. Nous n'avons observé aucune disparition du fructose, du sorbose ni du maltose dans les mêmes conditions.

Une liste de substances qui oxydent le TPNH en présence d'un extrait de vésicules séminales de mouton est donnée au Tableau I. Cette liste comprend des corps assez divers ayant de 3 à 7 carbones et dont certains sont des oses typiques et d'autres des substances apparentées. La plupart d'entre eux sont porteurs d'une fonction pseudo-

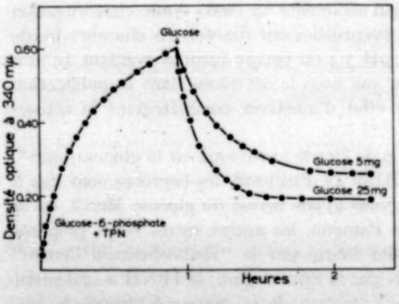

Fig. 1. Oxydation du TPNH par le glucose. Le mélange contient 0.2 ml d'extrait dialysé de vésicules séminales de mouton, 0.2 μmole de glucose-6-phosphate, 0.5 μmole de TPN et 50 μmoles de tampon phosphate à pH 7 dans un volume total de 3 ml. Le glucose est ajouté sous forme sèche. La réaction se passe à 18°.

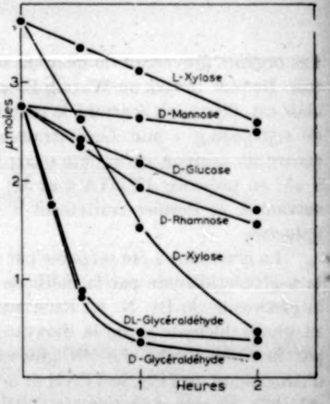

Fig. 2. Disparition de différents aldoses, couplée à l'oxydation du glucose-6-phosphate. 4 μmoles de glucose-6-phosphate, 0.2 μmole de TPN, 25 μmoles de tampon phosphate à pH 7 et 0.25 ml d'extrait dialysé de vésicules séminales traité par l'EDTA sont incubés à 37° en présence du substrat. Celui-ci est dosé par son pouvoir réducteur.

TABLEAU I

SUBSTRATS DE L'ALDOSE-RÉDUCTASE

Tous les essais enzymatiques ont été réalisés par la méthode spectrophotométrique avec un même extrait de vésicules séminales de mouton en présence d'EDTA et à trois concentrations différentes en substrat (deux seulement pour les heptoses). La constante de Michaëlis (K_M) et la vitesse maxima (V) ont été calculées par la méthode de LINEWEAVER ET BURK[13]. V est exprimé en % de la valeur trouvé pour la glucosone. Les valeurs entre parenthèses ont été obtenues avec une autre préparation enzymatique. Les chiffres ci-dessous sont destinés à donner l'ordre de grandeur des constantes plutôt qu'une valeur exacte.

Substrat	*V*	*K_M approximatif (moles/l)*
D-Glycéraldéhyde	110	$5 \cdot 10^{-4}$
L-Arabinose	56	$1.5 \cdot 10^{-3}$ $(3 \cdot 10^{-3})$
D-Ribose	55	$2 \cdot 10^{-3}$
D-Xylose	56	$1.5 \cdot 10^{-3}$
L-Xylose	56	$3 \cdot 10^{-2}$
D-Galactose	43	$3 \cdot 10^{-3}$
D-Glucose	51	$2 \cdot 10^{-2}$
D-Mannose	60	$5 \cdot 10^{-2}$
D-Glucosone	100	$4 \cdot 10^{-4}$
D-Glucosamine	30	$5 \cdot 10^{-2}$
D-Glucuronolactone*	70	$3 \cdot 10^{-4}$
L-Fucose	46	$2 \cdot 10^{-2}$
D-Rhamnose	38	$7 \cdot 10^{-3}$
D-Glycéro-L-alloheptose	19	$3 \cdot 10^{-2}$
D-Glycéro-D-galactoheptose	30	10^{-2}
D-Glycéro-L-galactoheptose	33	$5 \cdot 10^{-2}$
D-Glycéro-D-guloheptose	26	$2 \cdot 10^{-2}$
D-Glycéro-L-mannoheptose	17	$3 \cdot 10^{-2}$

* L'acide glucuronique réagit à peu près à la même vitesse que sa lactone.

aldéhydique mais quelques uns sont des aldéhydes vraies. En dépit du manque de pureté de la préparation enzymatique, nous avons dans chaque cas fait une mesure approximative de la constante de Michaëlis et de la vitesse maxima; on voit que l'enzyme présente une affinité très variable pour ses différents substrats et en a notamment 100 fois plus pour les glycéraldéhydes que pour le glucose; ce dernier fait pourrait indiquer que la réaction se fait avec la forme aldéhydique des oses. Les résultats de la Fig. 2 montrent aussi que la forme L de la glycéraldéhyde est réduite au même titre que son isomère D, car beaucoup plus de 50 % du mélange racémique a réagi au cours de cette expérience.

Les extraits de vésicules séminales de mouton catalysent aussi l'oxydation du TPNH par une série d'aldéhydes aliphatiques ou cycliques, notamment par les aldéhydes acétique, propionique et isobutyrique et par la benzaldéhyde. La cinétique de ces réactions n'a pas été étudiée.

Notons finalement que le DPNH ne peut remplacer le TPNH dans les réactions décrites ci-dessus et que la cétose-réductase, qui est présente dans les extraits de vésicules séminales, a été, au cours de ces expériences, inactivée par l'EDTA[1] et ne peut donc interférer dans nos essais.

2. *Produit de réduction des aldoses et réversibilité de la réaction*

Nous avons pu vérifier dans plusieurs cas que l'oxydation du TPN est effectivement couplée à la réduction de la fonction aldéhydique des oses. Nous avons déjà vu plus haut (Fig. 2) que ceux-ci perdent leur propriété réductrice au cours de la

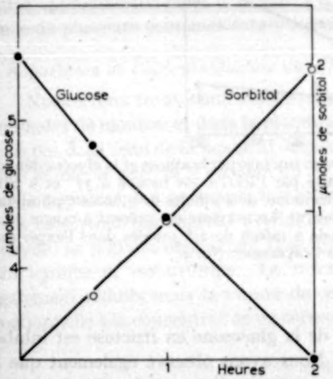

Fig. 3. Transformation quantitative du glucose en sorbitol par un extrait de placenta de mouton. 0.4 ml d'extrait placentaire dialysé, incubé à 37° et à pH 7 (tampon phosphate) en présence de 5.45 µmoles de glucose, de 4 µmoles de glucose-6-phosphate et de 0.5 µmole de TPN, dans un volume total de 0.75 ml. Le glucose est dosé par son pouvoir réducteur.

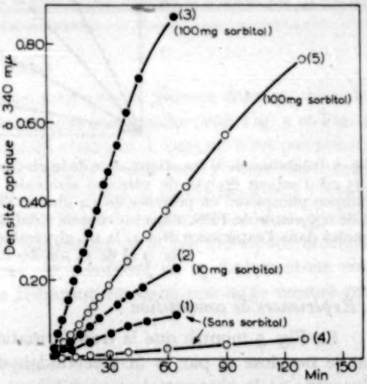

Fig. 4. Réversibilité de l'aldose-réductase. Le mélange d'incubation contient 0.5 µmole de TPN, 10 µmoles de tampon tris-hydroxyméthyl-aminométhane à pH 9.2, 0.5 ml d'extrait tissulaire dialysé et traité par l'EDTA (●, vésicules séminales de mouton; ○, placenta de vache) et du sorbitol comme indiqué. Le volume total est de 3 ml. La quantité de glucose formé à partir du sorbitol était de 0.05 µmole pour le No. 2 (TPNH formé: 0.058 µmole), 0.4 µmole pour le No. 3 (TPNH formé: 0.37 µmole) et 0.36 µmole pour le No. 5 (TPNH formé: 0.34 µmole).

réaction; ce n'est cependant pas le cas de la glucosone qui est transformée en fructose (voir Fig. 5). La formation de sorbitol à partir de glucose a été démontrée chromatographiquement (voir Fig. 2 de la publication suivante). La Fig. 3 montre en outre que la transformation du glucose en sorbitol par un extrait de placenta de mouton est quantitative.

La réversibilité de la réaction a été démontrée au cours de deux expériences, décrites à la Fig. 4, où du sorbitol et du TPN ont été incubés à pH 9.2 en présence d'un extrait de vésicules séminales de mouton ou de placenta de vache; du glucose, a été formé en quantités équimoléculaires à celles de TPN réduit.

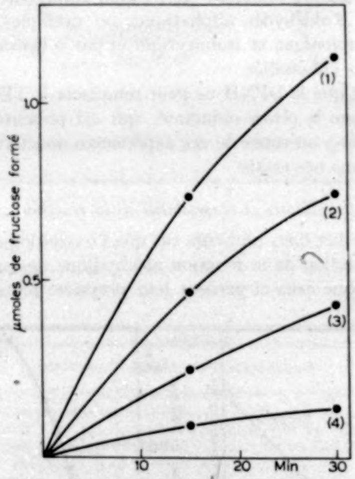

Fig. 5. Inhibition de la transformation de la glucosone en fructose par le xylose et la glycéraldéhyde. 0.25 ml d'extrait dialysé de vésicules séminales traité par l'EDTA est incubé à 37° et à pH 7 (tampon phosphate) en présence de 2.4 µmoles de glucosone, de 4 µmoles de glucose-6-phosphate et de 0.5 µmole de TPN, dans un volume total de 0.6 ml. Le D-xylose est présent à raison de 17 µmoles dans l'expérience No. 2, la DL-glycéraldéhyde à raison de 2.8 µmoles dans l'expérience No. 3 et de 14 µmoles dans l'expérience No. 4.

3. *Expériences de compétition*

La Fig. 5 montre que la transformation de la glucosone en fructose est inhibée par le D-xylose et par la DL-glycéraldéhyde. Nous avons observé également que la glucosone et la glucuronolactone inhibent presque complètement la transformation du glucose en sorbitol et en fructose par les extraits de vésicules seminales.

4. *Influence de l'ion* SO_4^{--} *et essais de purification*

L'enzyme présent dans les vésicules séminales est activé par l'ion sulfate, ajouté sous forme de Na_2SO_4 ou de $(NH_4)_2SO_4$. L'activité maximum est obtenue pour une concentration de 0.4 *M*. Les résultats qui figurent au Tableau II montrent que le degré d'activation n'est pas le même pour tous les substrats.

Les différents essais que nous avons faits n'ont abouti qu'à une purification très

TABLEAU II

INFLUENCE DES IONS SO_4^{--} SUR L'ALDOSE-RÉDUCTASE

La vitesse de la réaction est mesurée spectrophotométriquement. La préparation enzymatique est constituée par un extrait de vésicules séminales de mouton. Les aldoses sont présents en concentrations variables, d'autant plus grande que leur affinité pour l'enzyme est faible. Le sulfate est ajouté sous forme de sel d'ammonium à une concentration finale de 0.4 *M*.

Substrat	*% d'activation*
DL-Glycéraldéhyde	150
L-Arabinose	150
D-Ribose	150
D-Xylose	100
L-Xylose	70
D-Glucosone	150
D-Glucuronolactone	150
D-Glucose	20
D-Galactose	55
D-Mannose	15
L-Rhamnose	35
D-Glycéro-D-galactoheptose	25
D-Glycéro-D-guloheptose	25

peu importante de l'aldose-réductase. L'enzyme est soluble en présence de sulfate ammonique à 55% de saturation, il est détruit par chauffage à 55° mais est protégé contre cette dénaturation par le TPN. Un certain degré de purification a aussi été obtenu par adsorption des protéines inactives sur charbon (Darco) ou sur diaminoéthylcellulose. Au cours de ces essais, l'activité de l'enzyme sur les divers aldoses est restée approximativement la même et nous n'avons pas obtenu d'indication de l'existence de plusieurs protéines catalytiquement actives.

5. *Répartition de l'aldose-réductase dans les tissus*

Nous avons trouvé une aldose-réductase active sur le glucose dans les vésicules séminales de mouton et dans le placenta de mouton et de vache (voir Fig. 3 et Fig. 4) mais pas dans celui de la lapine ni dans le placenta humain. L'enzyme n'est pas présent dans le foie, le rein, ni le cerveau du rat. Les extraits de foie sont notamment inactifs sur le D-glucose, le D-mannose, le D-galactose, le D-ribose, le L-arabinose et le L-xylose mais catalysent par contre l'oxydation du TPNH par la D-glucosone, la DL-glycéraldéhyde, la D-glucuronolactone (Fig. 6), la benzaldéhyde et les aldéhydes acétique, propionique et isobutyrique. Le D-fructose, le L-sorbose et la dioxyacétone sont également réduits mais la vitesse de cette réduction est dans une large mesure proportionnelle à la concentration en cétoses.

DISCUSSION

Les extraits de vésicules séminales catalysent la réduction par le TPNH de presque tous les aldoses et aldéhydes testés. Le système enzymatique responsable de ces réactions n'a cependant pas été suffisamment purifié pour que nous puissions attribuer avec certitude l'ensemble des phénomènes observés à un seul enzyme. Nous proposons de conserver le nom d'"aldoses-réductase" à l'enzyme présent dans les vésicules séminales et le placenta de mouton et qui est responsable de la réduction du glucose en sorbitol et de réserver le nom d'"aldéhyde réductase" pour celui qui réduit notamment la benzaldéhyde et qui est particulièrement actif dans les extraits hépatiques.

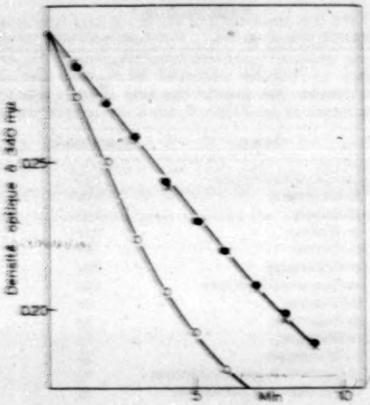

Fig. 6. Oxydation du TPNH par divers aldoses en présence d'extrait de foie. 0.1 ml de la fraction soluble d'un homogénat à 20% de foie de rat incubé à 28° en présence de 0.3 μmole de DL-isocitrate, 0.5 μmole de TPN, 0.5 μmole de $MgCl_2$ et 50 μmoles de tampon phosphate à pH 7.4. Lorsque l'oxydation de l'isocitrate est complète on ajoute 4 μmoles de D-glycéraldéhyde (○) ou 3 μmoles de DL-glycéraldéhyde (●) ou 3 μmoles de glucosone (×). Volume total 3 ml.

REMERCIEMENTS

Ce travail a été subsidié par le Centre National de Recherches Enzymologiques, l'Institut Interuniversitaire de Sciences Nucléaires et les Lilly Research Laboratories.

RÉSUMÉ

Les vésicules séminales et le placenta de mouton contiennent un système enzymatique, appelé aldose-réductase, qui catalyse la réduction par le TPNH d'un grand nombre d'aldoses ou de substances apparentées et de plusieurs aldéhydes aliphatiques ou cycliques. Le glucose est notamment réduit en sorbitol et la glucosone en fructose. Le foie de rat contient un système enzymatique analogue mais inactif sur le glucose et certains pentoses et pour lequel le nom d'"aldéhyde-réductase" a été suggéré.

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LE MÉCANISME DE LA FORMATION DU FRUCTOSE SÉMINAL ET DU FRUCTOSE FOETAL

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(Reçu le 1 avril 1959)

SUMMARY

Mechanism of the formation of seminal fructose and foetal fructose

1. Slices of sheep seminal vesicles irreversibly convert glucose to sorbitol and sorbitol to fructose. The fructose formed from [1-^{14}C]glucose is labelled only in position 1.

2. The same conversions are catalysed by extracts of seminal vesicles in the presence of glucose-6-phosphate, TPN and DPN.

3. The conversion of glucose to fructose and sorbitol by slices or extracts is inhibited by glucosone and glucuronolactone. The conversion of sorbitol to fructose is inhibited by ethylenediaminetetraacetate, which inactivates the ketose reductase.

4. These observations as well as the presence of sorbitol in the fresh gland indicate that the conversion of glucose to fructose by seminal vesicles takes place according to the reactions:

$$\text{Aldose reductase: Glucose} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{Sorbitol} + \text{TPN}^+$$
$$\text{Ketose reductase: Sorbitol} + \text{DPN}^+ \rightleftharpoons \text{Fructose} + \text{DPNH} + \text{H}^+$$
$$\text{Sum: Glucose} + \text{TPNH} + \text{DPN}^+ \rightleftharpoons \text{Fructose} + \text{TPN}^+ + \text{DPNH}$$

5. The presence of the aldose reductase in the placenta of the ewe, of the ketose reductase in the liver of the foetus and of sorbitol in the foetal blood indicates that the foetal fructose can be formed by the same mechanism, glucose being converted to sorbitol in the placenta and sorbitol to fructose in the liver.

6. The various mechanisms by which glucose could be converted to fructose are discussed.

INTRODUCTION

Dans le règne animal, le fructose se trouve à l'état naturel dans le sperme de la plupart des animaux[1] ainsi que dans le sang foetal des ongulés et de quelques autres groupes[2].

Le parallélisme observé par MANN ET PARSONS[3] entre la glycémie et la concentration du fructose séminal suggère fortement que ce dernier est formé aux dépens du glucose sanguin. Cette transformation aurait lieu dans les glandes annexes du tractus

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[*] Associé du F.N.R.S.

génital mâle, le plus souvent dans les vésicules séminales. Quant au fructose foetal, les expériences de Huggett et coll.[4,5] et Alexander et coll.[6,7] tout comme celles de Karvonen[8] indiquent qu'il est formé par le placenta à partir du glucose maternel.

La concentration du fructose dans le plasma séminal étant plusieurs fois supérieure à celle du glucose sanguin, la transformation de l'aldose en cétose ne peut s'expliquer par l'action d'une isomérase du type de celle décrite récemment par Marshall et Kooi[9] dans *Pseudomonas hydrophila*, mais doit nécessairement être couplée à une réaction exergonique. D'après Mann et Lutwak-Mann[10], le glucose serait phosphorylé en glucose-6-phosphate aux dépens de l'ATP* et l'hydrolyse enzymatique du fructose-6-phosphate formé sous l'action de la phosphohexo-isomérase donnerait naissance au fructose. Nous avons nous même proposé un autre mécanisme où le transfert d'électrons du TPNH sur le DPN$^+$ permet la transformation du glucose en sorbitol et du sorbitol en fructose[11,12]. Nous publions ici en détail les faits qui militent en faveur de l'intervention de ce processus dans la formation du fructose séminal et du fructose foetal.

TECHNIQUES EXPÉRIMENTALES

La plupart des techniques expérimentales on été décrites dans la publication précédente[13]. Le glucose uniformément marqué avec du ^{14}C ([^{14}C]glucose), le [1-^{14}C]glucose et le [1-^{14}C]sorbitol ont été fournis par le "Radiochemical Centre" d'Amersham. La γ et la δ gluconolactone sont un don du Professeur W. Z. Hassid. La sorbitol-déshydrogénase a été purifiée 25 fois à partir d'homogénats de foie de rat par fractionnement au moyen de sulfate ammonique entre 40 et 60 % de saturation, suivi d'un traitement au chloroforme–éthanol selon Blakley[14].

L'analyse de la distribution du ^{14}C dans la molécule de fructose a été réalisée par fermentation hétérolactique[15] après transformation en glucose. Pour ce faire, le fructose a été incubé en présence d'ATP et de MgCl$_2$ et d'un mélange d'hexokinase et de phosphohexo-isomérase (fraction 3a) préparé à partir de levures selon Berger et coll.[16]. Lorsque la réaction est terminée, on ajoute une suspension de microsomes de foie de rat, qui, grâce à leur activité glucose-6-phosphatasique très marquée, catalysent la transformation presque quantitative des hexoses-phosphates en glucose. Le mélange est finalement déprotéinisé par addition de Ba(OH)$_2$ et de ZnSO$_4$ et le glucose est repurifié par chromatographie sur papier dans le phénol saturé d'eau.

RÉSULTATS

1. *Transformation du glucose en sorbitol et en fructose par des tranches de vésicules séminales de mouton*

Des tranches de vésicules séminales de mouton transforment le glucose en un mélange de sorbitol et de fructose et oxydent le sorbitol en fructose mais non en glucose; ceci a pu être démontré grâce à l'emploi de [^{14}C]glucose et de [^{14}C]sorbitol et séparation chromatographique des sucres radioactifs présents à la fin de l'incubation (Fig. 1). Au cours d'autres expériences analogues et non représentées ici, on n'a pas observé de formation de glucose ni de sorbitol à partir de fructose marqué, bien que celui-ci ait donné naissance à du $^{14}CO_2$ en quantités comparables à celles qui sont formées à partir de glucose radioactif dans les mêmes conditions.

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* Nous utilisons les abréviations suivantes: ATP pour adénosinetriphosphate, DPN pour diphosphopyridine-nucléotide, TPN pour triphosphopyridine-nucléotide, EDTA pour éthylènediaminetétraacétate.

Les tranches de vésicules séminales catalysent donc la suite des transformations irréversibles:

Glucose → Sorbitol → Fructose

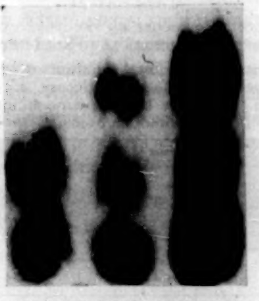

Fig. 1. Transformation du glucose en sorbitol et fructose par des tranches de vésicules séminales de mouton. Des tranches (150 mg) de vésicules séminales de mouton sont agitées pendant 2 h à 37° et en présence d'oxygène dans 0.25 ml de tampon phosphate selon KREBS ET HENSELEIT[17] contenant 5 μC de [1-^{14}C]glucose ou 1 μC de [^{14}C]glucose ou 1 μC de [1-^{14}C]sorbitol. Les sucres sont ensuite isolés et chromatographiés dans le phénol saturé d'eau. Le papier est séché et appliqué contre un film radiographique. La figure ci-dessus montre la séparation du glucose, du sorbitol et du fructose (de haut en bas). L'expérience de droite a été réalisée avec le [1-^{14}C]glucose, celle du milieu avec le [^{14}C]glucose et celle de gauche avec le [1-^{14}C]sorbitol.

L'examen de la Fig. 1 montre en outre que le [1-^{14}C]glucose donne naissance à du fructose marqué, au même titre que le [^{14}C]glucose. Ce fructose a été élué du papier et rechromatographié et la distribution du ^{14}C dans les 6 atomes de carbone a été déterminée. Comme le montre le Tableau I, la totalité de la radioactivité était localisée au premier carbone, ce qui suggère que la transformation du glucose en fructose a été réalisée sans scission de la molécule hydrocarbonée. Nous avons également vérifié que le glycérol radioactif n'est pas transformé en fructose par des tranches de vésicules séminales.

TABLEAU I

RÉPARTITION DU ^{14}C SUR LE FRUCTOSE FORMÉ A PARTIR DE [1-^{14}C]GLUCOSE

Les résultats sont exprimés en % de la radioactivité totale mesurée par oxydation complète de l'échantillon par le persulfate.

Carbone	*1*	*2*	*3*	*4*	*5*	*6*	*Récupération*
Radioactivité	106	0.5	0.5	0.1	0.1	0.1	107.3%

2. *Transformation du glucose en sorbitol et en fructose par des extraits de vésicules séminales*

Le Tableau II montre qu'un extrait dialysé de vésicules séminales de mouton transforme le glucose en fructose en présence de TPN, de DPN et de glucose-6-phosphate. Ce dernier ne donne naissance par lui-même qu'à de petites quantités de fructose et à des quantités deux fois plus élévées de glucose qui paraissent donc être formées par hydrolyse des hexoses-6-phosphates. Etant donné la présence de glucose-6-phosphate-déshydrogénase dans les extraits utilisés[13], il semble légitime d'attribuer au glucose-6-phosphate le rôle de donneur d'électrons pour la réduction du TPN.

TABLEAU II

TRANSFORMATION DU GLUCOSE EN FRUCTOSE PAR DES EXTRAITS DIALYSÉS DE VÉSICULES SÉMINALES DE MOUTON

0.25 ml d'extrait dialysé de vésicules séminales de mouton est incubé pendant 2 h à 37° et pH 6.8 (tampon phosphate) en présence de 5 μmoles de glucose, 2.5 μmoles de glucose-6-phosphate (G6P), 0.5 μmole de TPN ou 0.5 μmole de DPN, comme indiqué ci-dessous. Les résultats sont exprimés en μmoles.

Substrat	*Coenzymes*	*Δ Glucose*	*Δ Fructose*
Glucose	—	0	0
	DPN + TPN	— 0.28	+ 0.08
G6P	—	+ 0.17	+ 0.10
	DPN + TPN	+ 0.22	+ 0.12
Glucose + G6P	—	+ 0.17	+ 0.11
	DPN + TPN	— 2.34	+ 0.61

Le chromatogramme représenté à la Fig. 2 montre que le fructose formé par les extraits a pris naissance à partir du glucose et que du sorbitol est formé simultanément, tandis qu'après inactivation de la cétose-réductase par l'EDTA, le sorbitol seul est formé.

Fig. 2. Transformation du glucose en sorbitol et fructose par des extraits de vésicules séminales de mouton. 0.25 ml d'extrait dialysé de vésicules séminales de mouton, incubé pendant 2 h à 37° en présence de 0.25 μmoles de [^{14}C]glucose (1 μC), 1 μmole de glucose-6-phosphate, 0.1 μmole de TPN et 50 μmoles de tampon phosphate à pH 7. Les taches correspondent au glucose, sorbitol et fructose (de haut en bas). L'expérience de droite a été réalisée en présence de 5 μmoles d'EDTA.

3. *La cétose-réductase des vésicules séminales de mouton*

Williams-Ashman et Banks[18], ayant observé la présence de la cétose-réductase (sorbitol-déshydrogénase) dans les glandes annexes du tractus génital mâle du rat, ont les premiers suggéré le rôle possible de cet enzyme dans la formation du fructose séminal. La Fig. 3 montre que la cétose-réductase est présente dans les vésicules séminales de mouton et qu'elle est inactivée par une incubation de quelques minutes en présence d'EDTA. Cette inactivation est cependant beaucoup plus lente une fois que la réaction enzymatique a débuté, ce qui suggère une protection de l'enzyme par un des produits de la réaction. La Fig. 4 montre que c'est effectivement le DPNH qui protège la cétose-réductase contre l'inactivation par l'EDTA. Rappelons que Williams-Ashman, Banks et Wolfson[19] ont récemment décrit une inhibition complète de la cétose-réductase que le dimercaptopropanol, tandis que Hoch et Vallee[20] ont observé que le DPN$^+$ protège l'alcool-déshydrogénase de levure contre l'inactivation par la 1,10-phénantroline.

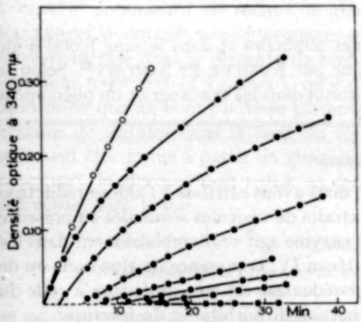

Fig. 3. Présence de la cétose-réductase dans les vésicules séminales de mouton et son inactivation par l'EDTA. 0.1 ml d'extrait dialysé de vésicules séminales de mouton incubé à 18° en présence de 0.5 μmole de DPN et 5 μmoles de tampon phosphate à pH 7. 10 μmoles d'EDTA sont ajoutées au début de l'expérience sauf dans un contrôle (○) et la réaction est déclenchée, après des temps variables, par addition de 5 μmoles de sorbitol. Volume total: 3 ml. Il a été vérifié qu'aucune inactivation ne se produit en l'absence d'EDTA.

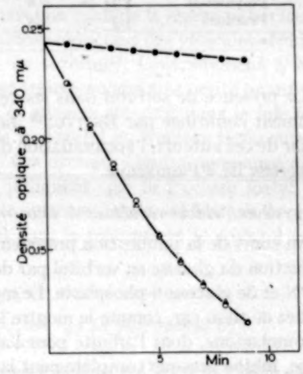

Fig. 4. Protection de la cétose-réductase par le DPNH. 0.1 ml d'extrait dialysé de vésicules séminales de mouton incubé à 18° en présence de 0.1 μmole de DPNH, 5 μmoles de fructose, et 5 μmoles de tampon tris-hydroxyméthyl-aminométhane à pH 7.4. Volume total: 3 ml. △ sans EDTA. ○ 10 μmoles d'EDTA ajoutées après le DPNH, 15 min avant le fructose. ● 10 μmoles d'EDTA ajoutées avec le fructose, 15 min avant le DPNH.

4. *Présence de sorbitol dans les vésicules séminales et le sang foetal du mouton*

Ainsi que nous l'avons décrit antérieurement[21], les vésicules séminales du mouton et le sang foetal du même animal contiennent une quantité importante d'un polyalcool qui n'est ni réducteur, ni fermentescible, ni retenu par les échangeurs d'ions et qui se confond chromatographiquement avec le sorbitol. Il est facilement mesuré par la méthode de Faulkner[22] et nous montrons au Tableau III sa concentration dans plusieurs échantillons des deux tissus, comparée à celle du fructose. Débarrassé des hexoses par fermentation et purifié par passage sur des échangeurs d'anions (Amberlite IR 45) et de cations (Amberlite IR 120) il ne réagit que partiellement avec le DPN en présence de cétose-réductase purifiée. D'après ces analyses, le sorbitol ne constitue pas plus de 50 % des valeurs représentées au Tableau III dans le cas des vésicules séminales et 25 % dans le cas du sang foetal.

TABLEAU III

PRÉSENCE D'UN POLYALCOOL DANS LES VÉSICULES SÉMINALES ET LE SANG FOETAL DU MOUTON

Le polyalcool est dosé par la méthode de FAULKNER[22], le sorbitol étant pris comme étalon. La concentration en fructose est donnée à titre de comparaison. Pour chaque tissu, nous donnons les résultats obtenus chez 3 animaux différents. Les concentrations sont exprimées en mg/g de tissu frais ou mg/ml de sang.

	Vésicules séminales			*Sang foetal*		
Polyalcool	4.35	2.8	2.78	0.42	0.49	0.90
Fructose	5.3	3.5	6.15	0.86	1.07	1.80

La présence de sorbitol dans les vésicules séminales et dans le sang foetal a été récemment confirmée par BRITTON[23] ainsi que par KUBICEK ET SANTAVY[24]. Selon le premier de ces auteurs, l'accumulation du sorbitol dans les tissus serait un phénomène *post-mortem* lié à l'anoxémie.

5. *Le système "aldose-réductase + cétose-réductase"*

Au cours de la publication précédente[13], nous avons attribué à l'aldose-réductase la réduction du glucose en sorbitol par des extraits de vésicules séminales en présence de TPN et de glucose-6-phosphate. Le même enzyme agit vraisemblablement dans les tranches de tissu car, comme le montre le Tableau IV, la présence de glucosone ou de glucuronolactone, dont l'affinité pour l'aldose-réductase est très supérieure à celle du glucose, inhibe presque complètement la formation du sorbitol et du fructose.

TABLEAU IV

INHIBITION DE LA TRANSFORMATION DU GLUCOSE EN SORBITOL ET FRUCTOSE PAR LA GLUCOSONE ET LA GLUCURONOLACTONE

Expérience réalisée avec 100 mg de tranches de vésicules séminales par une technique analogue à celle décrite à la Fig. 1. Le [^{14}C]glucose est ajouté à raison de 1 μC (0.3 μmole) dans un volume total de 0.3 ml. Le sorbitol et le fructose, étant incomplètement séparés au cours de la chromatographie, sont élués ensemble et une partie aliquote de la solution est séchée sur un disque d'aluminium et comptée.

Addition	*Radioactivité dans le sorbitol et le fructose, en % du contrôle*
—	100
Glucosone, 5 μmoles	5
Glucuronolactone, 5 μmoles	5

L'inhibition par l'EDTA de la formation du fructose par des extraits de vésicules séminales (Fig. 2) et l'oxydation facile du sorbitol en fructose par les tranches de tissu (Fig. 1) indiquent d'autre part la participation de la cétose-réductase à la transformation du glucose en fructose. Celle-ci se ferait donc par la suite des deux réactions suivantes:

$$\begin{array}{rl} \text{Aldose-réductase:} & \text{Glucose} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{Sorbitol} + \text{TPN}^+ \\ \text{Cétose-réductase:} & \text{Sorbitol} + \text{DPN}^+ \rightleftharpoons \text{Fructose} + \text{DPNH} + \text{H}^+ \\ \hline \text{Somme:} & \text{Glucose} + \text{TPNH} + \text{DPN}^+ \rightleftharpoons \text{Fructose} + \text{TPN}^+ + \text{DPNH} \end{array}$$

Dans ce système, la transformation du glucose en fructose est donc couplée à la réduction du DPN⁺ par le TPNH et, bien que les deux réactions soient réversibles, l'irréversibilité que nous avons observée peut s'expliquer par le fait que, selon GLOCK et MCLEAN[25], la presque totalité du TPN tissulaire se trouve sous sa forme réduite, tandis que le DPN se trouve principalement sous sa forme oxydée.

6. *Présence de l'aldose-réductase et de la cétose-réductase dans le tissu embryonnaire du mouton*

Le placenta de brebis et celui de vache, mais non celui de la lapine ni le placenta humain, contiennent un enzyme analogue à l'aldose-réductase des vésicules séminales et capable notamment de réduire le glucose en sorbitol[13]. Contrairement à NEIL, WALKER ET WARREN[26], nous n'avons cependant trouvé qu'une très petite quantité de cétose-réductase dans le placenta de brebis. Cet enzyme est actif dans le foie foetal dont nous montrons au Tableau V qu'il oxyde le sorbitol en fructose beaucoup plus rapidement que ne le fait le tissu placentaire. Ces diverses observations ainsi que la présence de sorbitol dans le sang du foetus indiquent que le fructose foetal peut également être formé à partir du glucose par le système "aldose-réductase + cétose-réductase", le glucose étant réduit en sorbitol dans le placenta et le sorbitol oxydé en fructose dans le foie.

TABLEAU V

TRANSFORMATION DU SORBITOL EN FRUCTOSE PAR LE FOIE FOETAL ET LE PLACENTA DE MOUTON

250 mg de tissu finement coupé aux ciseaux, sont agités pendant 1 h à 37° dans 1 ml de tampon phosphate selon KREBS ET HENSELEIT[17].

Exp.	*Sorbitol ajouté*	*Fructose formé (μmoles/g)*	
		Foie foetal	*Placenta*
1	0	0.5	0.5
	50 μmoles	14.0	0.43
2	0	0	0.1
	50 μmoles	7.8	0.43

7. *Rôle possible de la glucosone dans la transformation du glucose en fructose*

La très grande facilité avec laquelle l'aldose-réductase forme du fructose à partir de glucosone nous amène à examiner l'éventualité du mécanisme suivant:

$$\text{Glucose} \xrightarrow{-2\text{H}} \text{Glucosone} \xrightarrow{\text{TPNH}} \text{Fructose}$$

Tous les essais que nous avons fait pour vérifier cette hypothèse ont cependant donné des résultats négatifs. Aucune trace de glucosone radioactive n'a pu être décelée chromatographiquement dans les produits du métabolisme du $[^{14}C]$glucose par des tranches de vésicules séminales, même lorsque de grandes quantités de glucosone non marquée étaient préalablement ajoutées au milieu d'incubation. D'autre part, bien que nous ayons observé dans les vésicules séminales la présence de la glucose-déhydrogénase, dont on sait qu'elle oxyde le glucose en δ-gluconolactone, isomère de la glucosone, ni cette lactone, ni la γ-gluconolactone, ne donnent naissance à du fructose lorsqu'on les incube avec des extraits de tissus. La glucosone ne réagit pas non plus avec le DPNH, ce qui devrait être le cas si les vésicules séminales contenaient une glucose-déshydrogénase susceptible de lui donner naissance. Nous devons donc provisoirement en conclure que la réduction très rapide de la glucosone en fructose par l'aldose-réductase n'a pas de signification physiologique et reflète simplement le spectre d'action assez étendu de l'enzyme.

8. *Etude des phosphatases des vésicules séminales et du placenta*

KELLERMAN[27] a récemment présenté des résultats suggérant la présence d'une fructose-6-phosphatase spécifique dans les vésicules séminales du cobaye. Opérant dans des conditions identiques aux siennes, nous n'avons pu reproduire ses résultats et nous montrons au Tableau VI que l'hydrolyse du fructose-6-phosphate par un extrait de vésicules séminales de cobaye n'est nullement préférentielle à celle du glycérophosphate ou du glucose-6-phosphate. Nous avons de même toujours échoué dans les diverses tentatives que nous avons faites pour déceler la présence, dans les vésicules séminales et le placenta de mouton, de phosphatases spécifiques du fructose-6-phosphate, du fructose-1-phosphate ou du sorbitol-6-phosphate[12].

TABLEAU VI

ÉTUDE DE LA PHOSPHATASE ACIDE DES VÉSICULES SÉMINALES DE COBAYE

0.1 ml d'un homogénat à 5% de muqueuse de vésicules séminales de cobaye, est incubé pendant 30 min à 37° en présence de tampon acétate à pH 4.65 et du substrat. Il a été vérifié que le glucose-6-phosphate n'est pas transformé en fructose-6-phosphate au cours de ces expériences.

Substrat	*µmoles de P libéré par g de tissu et par heure*	
	— NaF	*+ NaF 0.01 M*
Fructose-6-phosphate	400	0
Glucose-6-phosphate	420	0
β-Glycéro-phosphate	1000	0
Fructose-6-P + glucose-6-P	503	—
Fructose-6-P + β-glycéro-P	1040	—
Glucose-6-P + β-glycéro-P	980	—

DISCUSSION

1. *Transformation du glucose en fructose par un mécanisme de phosphorylation suivie de déphosphorylation*

Nous avons signalé plus haut l'hypothèse émise par MANN ET LUTWAK-MANN[10] et selon laquelle le fructose séminal serait formé par hydrolyse des hexoses-phosphates tissulaires sous l'action d'une phosphatase alcaline, tandis que le glucose qui apparaît simultanément serait réutilisé sélectivement. Les faits expérimentaux à la base de cette hypothèse sont une utilisation glycolytique plus rapide du glucose que du fructose par les vésicules séminales du rat [28] et l'existence d'une phosphatase alcaline dans les mêmes organes du taureau et du rat. Plus récemment, KELLERMAN[27] a montré la présence, dans les vésicules séminales du cobaye, d'une hexokinase non spécifique dont l'action sur le fructose est inhibée compétitivement par le glucose.

L'hypothèse avancée par MANN ET LUTWAK-MANN n'a cependant reçu aucune confirmation directe et sa principale faiblesse réside dans le fait qu'elle fait intervenir des enzymes qui existent dans beaucoup de tissus et n'explique pas, par conséquent, la localisation très particulière de la synthèse du fructose dans l'organisme animal.

2. *Transformation du glucose en fructose par l'intermédiaire des trioses ou de la glucosone*

Le fait que le fructose formé à partir de [1-^{14}C]glucose était marqué uniquement en position 1 élimine un certain nombre de mécanismes possibles faisant intervenir une scission de la molécule d'hexose. La possibilité que le glycérol soit un intermédiaire dans la transformation du glucose en sorbitol ou en fructose a également été écartée. La glucosone est facilement réduite en fructose mais aucune transformation du glucose en glucosone n'a pu être démontrée.

3. *Transformation du glucose en fructose par le système "aldose-réductase + cétose-réductase"*

Nous pouvons résumer comme suit les principaux faits qui militent en faveur de ce mécanisme.

(1) Jusqu'à présent, la présence d'une aldose-réductase active sur le glucose n'a été observée que dans les vésicules séminales de mouton et dans le tissu embryonnaire d'animaux dont le sang foetal contient du fructose. La présence de la cétose-réductase dans les mêmes tissus complète donc ce système enzymatique dont la distribution dans l'organisme correspond à celle de la transformation du glucose en fructose.

(2) Les tranches de vésicules séminales de mouton transforment irréversiblement le glucose en sorbitol et le sorbitol en fructose. Cette observation a été confirmée par BRITTON[23] qui croit cependant que la cellule glandulaire pourrait être imperméable au fructose. Cette interprétation est contredite par le fait que le fructose est facilement oxydé par la même préparation.

(3) La transformation du glucose en sorbitol et fructose par les tranches de vésicules séminales est presque complètement inhibée par la glucosone et la glucuronolactone, qui ont beaucoup plus d'affinité que le glucose pour l'aldose-réductase.

(4) Des extraits dialysés du même tissu réalisent les mêmes transformations que les tranches pour autant qu'on les enrichisse en TPN et DPN et en glucose-6-phosphate dont nous avons montré qu'il réduit le TPN$^+$ en TPNH. L'EDTA inactive la cétose-réductase et empêche la formation de fructose mais non celle de sorbitol à partir de glucose.

(5) La présence de sorbitol dans les vésicules séminales et le sang foetal, tout en étant en accord avec le mécanisme que nous proposons, pourrait être comme BRITTON[23] le suggère, un phénomène post-mortem. Il nous parait en effet vraisemblable qu'en l'absence d'oxygène la réduction du glucose en sorbitol par le TPNH se poursuive, étant donné qu'elle ne nécessite que la présence de glucose-6-phosphate, tandis que l'oxydation du sorbitol en fructose sera arrêtée.

(6) Ainsi que nous le discutons ci-dessous, les caractéristiques thermodynamiques

du mécanisme que nous proposons sont en accord avec l'irréversibilité de la transformation du glucose en fructose.

4. *Considérations thermodynamiques*

L'observation faite par Glock et McLean[25] que le rapport $\frac{[TPN^+]\,[DPNH]}{[TPNH]\,[DPN^+]}$ est très petit dans la plupart des tissus nous permet de comprendre l'irréversibilité de la transformation du glucose en fructose par des tranches de vésicules séminales aussi bien que l'accumulation physiologique du fructose dans le même tissu. Le haut degré de réduction du TPN doit probablement être mis en relation avec la faible activité de la TPNH-cytochrome *c*-réductase, comparée à celle de la réductase spécifique du DPNH[29, 30] et aussi avec le fait que la déshydrogénation du glucose-6-phosphate, couplée à la réduction TPN, est rendue pratiquement complète par l'hydrolyse de la 6-phosphogluconolactone.

Le degré fort différent de réduction des deux coenzymes permet de prédire que, *in vivo*, la réduction d'un substrat sera beaucoup plus complète si le donneur d'électrons est le TPNH plutôt que le DPNH, tandis que les oxydations se feront le mieux à l'aide du DPN$^+$. Ces considérations mènent à la conclusion qu'un certain nombre de processus métaboliques, qui sont réversibles dans un système isolé, se font vraisemblablement à sens unique dans la cellule intacte. Ce sont plus particulièrement ceux qui, comme la transformation du glucose en fructose, s'accompagnent d'un transfert d'électrons du TPNH sur le DPN$^+$, car, si on admet que dans la cellule le rapport $\frac{[TPNH]\,[DPN^+]}{[TPN^+]\,[DPNH]}$ est égal à 20, le transfert d'électrons du TPNH sur le DPN$^+$ à 37° s'accompagne d'une diminution d'énergie libre de 1840 cal/mole. Nous pouvons citer par exemple: la biosynthèse de l'acide ascorbique qui débute par l'oxydation du glucose en glucuronolactone par le DPN$^+$, suivie de la réduction de la glucuronolactone en gulonolactone; la transformation du L-xylulose en D-xylulose via le xylitol[31] par les mitochondries de foie, celle du sorbose en fructose par *Acetobacter suboxydans*[32] et enfin la phosphorylation du pyruvate en phosphoénolpyruvate via le malate et l'oxalacétate.

5. *Métabolisme du fructose foetal*

Les expériences de Karvonen[8] ainsi que celles de Huggett, Warren et Winterton[4] ont montré que le foetus de mouton, séparé de son placenta, ne transforme pas le glucose en fructose, tandis que Neil, Walker et Warren[26] ont observé que le foetus isolé de chèvre oxyde rapidement le sorbitol en fructose mais que le placenta perfusé ne le fait pas. Ces faits sont parfaitement compatibles avec nos observations enzymologiques qui localisent la transformation du glucose en sorbitol dans le placenta et celle du sorbitol en fructose dans le foie foetal, tandis que le polyalcool est présent dans le sang. Huggett, Warren et Warren[5] ont de plus observé une augmentation de la concentration en fructose dans le liquide de perfusion qui traverse le placenta de mouton séparé de son foetus. Ce phénomène, dans la mesure où il reflète une réelle néoformation de fructose par le placenta, est plus difficilement interprétable à la lumière de nos résultats. On ne peut cependant exclure que le placenta à terme soit plus riche en cétose-réductase que ceux que nous avons étudiés.

L'utilisation du fructose par le foetus pose également un problème qui n'est pas encore résolu avec certitude. Neil et coll.[26] ont observé que le foetus de chèvre ne transforme pas le fructose en glucose. On sait d'ailleurs par des études faites chez le rat et le cobaye, que le foie foetal ne contient pas de glucose-6-phosphatase[33] ni de fructokinase[34] mais seulement une hexokinase non spécifique[35]. Nous avons nous même observé[36] qu'un extrait dialysé de foie de mouton foetal phosphoryle le fructose en présence d'ATP aussi bien que le glucose mais que cette réaction est complètement supprimée par la présence de glucose à concentration égale à celle du fructose. Le sorbitol n'est pas phosphorylé.

REMERCIEMENTS

Ce travail a été subsidié par le Centre National de Recherches Enzymologiques, l'Institut Interuniversitaire de Sciences Nucléaires et les Lilly Research Laboratories.

RÉSUMÉ

1. Des tranches de vésicules séminales de mouton transforment irréversiblement le glucose en sorbitol et le sorbitol en fructose. Le fructose formé à partir de [1-^{14}C]-glucose est marqué uniquement en position 1.

2. Les mêmes transformations sont catalysées par des extraits de vésicules séminales, en présence de glucose-6-phosphate, de TPN et de DPN.

3. La transformation du glucose en fructose et sorbitol par les tranches ou les extraits est inhibée par la glucosone et la glucuronolactone. Celle de sorbitol en fructose est inhibée par l'éthylènediaminetétraacétate, qui inactive la cétose-réductase.

4. Ces observations ainsi que la présence de sorbitol dans la glande fraîche indiquent que la transformation du glucose en fructose par les vésicules séminales se fait par la suite de réactions:

$$\begin{array}{rl} \text{Aldose-réductase:} & \text{Glucose} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{Sorbitol} + \text{TPN}^+ \\ \text{Cétose-réductase:} & \text{Sorbitol} + \text{DPN}^+ \rightleftharpoons \text{Fructose} + \text{DPNH} + \text{H}^+ \\ \hline \text{Somme:} & \text{Glucose} + \text{TPNH} + \text{DPN}^+ \rightleftharpoons \text{Fructose} + \text{TPN}^+ + \text{DPNH} \end{array}$$

5. La présence de l'aldose-réductase dans le placenta de la brebis, de la cétose-réductase dans le foie du foetus et du sorbitol dans le sang foetal montre que le fructose foetal peut être formé par le même mécanisme, le glucose étant transformé en sorbitol dans le placenta et le sorbitol en fructose dans le foie.

6. Les différents mécanismes par lesquels le glucose pourrait être transformé en fructose sont discutés.

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Short Communications

The use of paper chromatography for observing the effect of penicillin on the amino compounds present in sensitive and resistant strains of *Staphylococcus aureus*

During preliminary attempts to differentiate species of micrococci by a qualitative determination of their free amino constituents on paper chromatograms[1] it seemed possible that the technique could be used to demonstrate that penicillin-sensitive staphylococci, when treated with the drug, have a reduced ability to concentrate amino acids within the cell and also accumulate nucleotide-peptide complexes[2–5]. If the technique enabled the two effects to be observed simultaneously it could be useful for investigating the primary action of penicillin on these organisms.

Penicillin (0.1, 0.5 I.U./ml) was added to a shaken culture of a sensitive strain of *S. aureus* (N.C.T.C.* 8532) in its logarithmic phase and extracts of the washed cells were prepared for chromatography[1] after 1, 2 and 3-h contact with the drug (Fig. 1). One-dimensional chromatograms developed with the butanol–acetic acid solvent showed clearly that after 2 h the intensity of most of the amino acids and peptides in the penicillin-treated cells had decreased but an increase had occurred in the intensity of 2 compounds located just below the origin (referred to as Peptides 1 and 2) and of the origin. The increase in concentration of Peptide 1 (immediately below the origin)

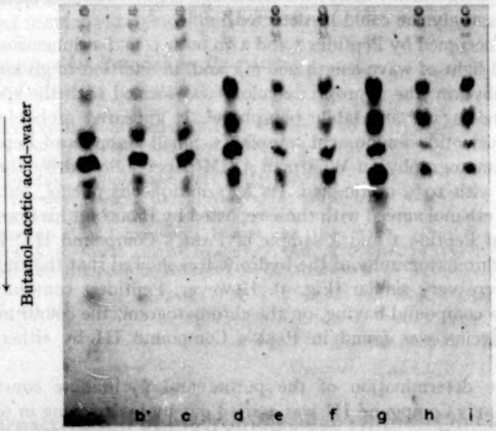

Fig. 1. One-dimensional chromatograms prepared from *S. aureus* showing the effect of penicillin treatment on the amino acids and peptides in the cell. a, d, g, control cultures after 0, 2, 3 h. b, e, h, cultures treated with 0.1 I.U. penicillin/ml and extracted after 0, 2, 3 h. c, f, i, cultures treated with 0.5 I.U. penicillin/ml and extracted after 0, 2, 3 h. Spray, ninhydrin.

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* N.C.T.C. National Collection of Type Cultures, Colindale, London.
Abbreviation: I.U., International unit.

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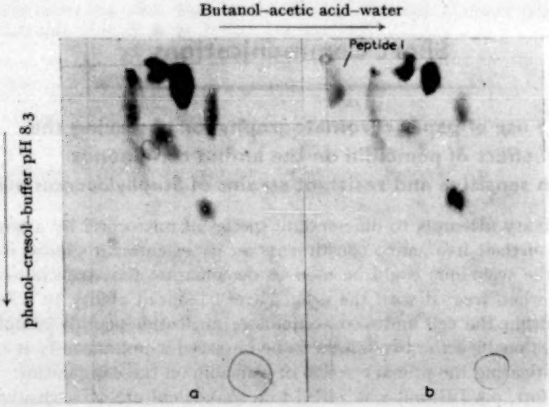

Fig. 2. Two-dimensional chromatograms prepared from *S. aureus* (a) untreated, (b) treated with penicillin (0.5 I.U./ml) for 2 h. Spray, ninhydrin.

was particularly noticeable. When 2-dimensional chromatograms were prepared from the same extracts, Peptide 1 was found to streak in the phenol–cresol solvent and the sharp decrease in amino acid concentration was confirmed (Fig. 2). Chromatograms prepared from cells which had been in contact with penicillin for 1 h also showed an increase in Peptide 1 and in material at the origin. Changes in amino acid concentration, however, were much less obvious, although slight decreases in the intensity of glutamic acid and glycine could be detected.

The position occupied by Peptides 1 and 2 on both 1- and 2-dimensional chromatograms absorbed light of wave length 260 mμ and, in addition to giving a positive reaction with ninhydrin, the appropriate colour was formed with the specific spray reagents for amino-sugars[6] and labile phosphate[7]. It appeared probable, therefore, that they were nucleotide–amino acid complexes. Small amounts of Peptide 1 were prepared by chromatography on Whatman 3 MM paper followed by elution of the appropriate area with 10% acetic acid. Its R_F's in aqueous phenol, butanol–acetic acid and aqueous ethanol agreed with those reported by PARK[3] for his Compound III. Also hydrolysis of Peptide 1 and a sample of PARK's Compound III[*] in 6 *N* HCl (105°, 16 h) and chromatography of the hydrolysates showed that the amino constituents of both were very similar (Fig. 3). However, Peptide 1 contained a higher concentration of a compound having, on the chromatogram, the colour and position of glycine. No glycine was found in PARK's Compound III by either PARK[3] or STROMINGER[4].

A qualitative determination of the purine and pyrimidine constituents of Peptide 1 and PARK's Compound III was carried out by hydrolysing in sealed tubes with trifluoracetic acid[8] (155° for 1 h) followed by 1-dimensional paper chromatography[9]. As reported by PARK, Compound III was found to contain uracil. In addition to uracil, however, Peptide 1 appeared to contain traces of adenine, although insufficient material was available for confirmation. Considering that Peptide 1 was prepared

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[*] Kindly supplied by J. T. PARK.

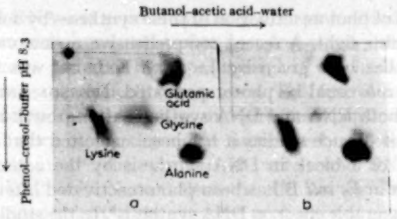

Fig. 3. Two-dimensional chromatograms of hydrolysates of (a) Peptide 1; (b) PARK's Compound III. Spray, ninhydrin.

by elution from an area of the chromatogram near the origin and examined without further purification its resemblance to PARK's Compound III is striking.

No detailed examination of Peptide 2 has been made. Its amino components were alanine with traces of methionine and isoleucine and it may therefore be identical with PARK's Compound II.

The increase in concentration of Peptide 1 after treatment with penicillin has also been demonstrated with 13 other sensitive strains of *S. aureus* of different phage types. As expected, however, 7 naturally resistant strains did not show the effect. Nor was the effect demonstrable with a resistant strain developed in the laboratory from N.C.T.C. 8532 by successive sub-culture in a medium containing increasing concentrations of penicillin. In this case, however, there was a striking decrease in the amino acid pool of the cell as resistance to the drug was acquired. This was in marked contrast to the high level of amino acids found in the naturally resistant strains which were examined and may reflect the difference between naturally acquired and laboratory-induced resistance to penicillin in *S. aureus*.

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Received April 6th, 1959

Biochim. Biophys. Acta, 37 (1960) 139–141

Photoreactivation of macromolecular synthesis in *Escherichia coli*

In the course of a quantitative study[1] of the effect of monochromatic u.v. light on protein and nucleic acid synthesis in *Escherichia coli* 15_{T-} it became of interest to

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

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look at the possibility of photoreactivation of these syntheses by subsequent exposure of the bacteria to visible light. A recent comprehensive review on the phenomenon of photoreactivation has been given by Jagger[2]. Kelner[3] was first to show that DNA synthesis in *E. coli* could be photoreactivated. Iverson and Giese[4] obtained photoreactivation of both RNA and DNA synthesis after growth-inhibitory doses of u.v. light. On the basis of such studies it has been suggested that photoreactivation involves the removal of a block in DNA synthesis by the action of visible light. However, cell division in *E. coli* B has been photoreactivated after low doses of u.v. light which had no measurable effect on DNA synthesis[5]. In the studies to be described, protein synthesis and RNA synthesis were photoreactivated under conditions of negligible DNA synthesis.

Escherichia coli 15_{T-} (kindly supplied by S. S. Cohen) were grown aerobically at 37° in a Tris-buffered synthetic medium containing a thymidine supplement[6]. Log-phase cells collected by centrifugation were resuspended in Tris buffer (pH 7.2) at 37° at a concentration of $2.5 \cdot 10^8$/ml for the irradiations with u.v. light in a water-prism monochromator[7]. High-intensity visible light was obtained with an apparatus constructed by R. A. Deering. Light from an air-cooled G. E. Hg AH-4 lamp was filtered by a 1/4-in. sheet of lead glass and a 1-cm layer of 2.5 % $CuCl_2$ to exclude wavelengths below 3100 Å and above 7500 Å respectively. An intensity of about $2.5 \cdot 10^6$ ergs/cm²/min was achieved at the position of the sample tube, suspended above the light source in a 37° water bath. Another sample from the same u.v. irradiation was also placed in this bath but shielded completely from the light. A control culture received neither u.v.-nor visible-light irradiation. The irradiated cells in buffer were exposed to visible light for a 10-min period beginning within 10 min after the u.v. irradiation. (A visible light dose of $2.5 \cdot 10^7$ ergs/cm² allows maximum photoreactivation of colony-forming ability in *E. coli* B.)[8] The visible-light dose had no evident effect on synthetic activity in an unirradiated control culture.

The treated cells were immediately resuspended in the complete growth medium at 37° and aeration was resumed. Synthesis of protein was followed by the incorporation of ^{35}S ($^{35}SO_4$ present in medium at 0.5 μC/ml) into the trichloroacetic acid fraction collected from the medium by the membrane-filter technique of Roberts *et al.*[9]. An adaptation of the Schmidt-Thannhauser procedure to the filter technique facilitated the following of RNA and DNA syntheses by the incorporation of ^{32}P into the respective fractions[6]. ($^{32}PO_4$ was present in medium at 0.5 μC/ml for these studies). The incorporation data was obtained on duplicate 0.2-ml samples taken from the cultures at 10-min intervals. The results have been confirmed by repetition of the experiments. Linear rates of synthesis were observed in both RNA and protein fractions after the doses of u.v. light used in these studies[1]. Accordingly, the results could be studied as a comparison of the different slopes of linear incorporation curves.

DNA synthesis was observed after the bacteria had received an average dose of 150 ergs/mm² at 2652 Å. This dose stopped DNA synthesis completely for a period of 40 min (division period, 50 min), after which period synthesis resumed at an exponential rate corresponding to that of the unirradiated control. When the visible light was administered after the u.v., the cells behaved as if they had received a u.v. dose of about 80 ergs/mm², resuming exponential DNA synthesis after about 30 min delay. The photoreactivation effect here was to reduce the delay before the resumption of DNA synthesis at the control rate.

The results on RNA and protein synthesis are summarized in Table I. The dose of u.v. light used was 200 ergs/mm^2 at 2652 Å, a dose which completely inhibits DNA synthesis for the entire period of the experiment, but which allows RNA and protein syntheses to continue at reduced linear rates. The figures in the table represent relative slopes of incorporation curves 35 min after resuspension of the cells in growth medium. In the absence of the thymidine supplement, which is required for DNA synthesis in this organism[10], the synthesis of protein and RNA continue at nearly linear rates for several hours. Nevertheless, the effect of a given dose of u.v. light on these syntheses is independent of the presence or absence of thymidine. Furthermore, it is apparent that photoreactivation of these syntheses can occur in the absence of DNA synthesis. ^{32}P-incorporation data indicated negligible DNA synthesis in the control culture in the absence of thymidine.

TABLE I

PHOTOREACTIVATION OF RNA AND PROTEIN SYNTHESIS IN *Escherichia coli* 15_T

	Thymidine present			*Thymidine absent*		
	Control	*u.v.*	*u.v.+visible*	*Control*	*u.v.*	*u.v. + visible*
RNA	1.00	0.56	0.60	1.00	0.55	0.78
Protein	1.00	0.26	0.30	1.00	0.24	0.31

It would appear that photoreactivation basically involves a repair of DNA *integrity* rather than a restoration of DNA *synthesis*. The "^{32}P-radiosuicide" studies of McFall *et al.*[11] have provided further evidence that bacterial RNA and protein syntheses depend on DNA *integrity* rather than DNA *synthesis*.

The fact that a higher degree of photoreactivation of RNA synthesis is obtained in the absence of thymidine could indicate that slightly damaged DNA may further damage itself in attempting to replicate. It should be mentioned that it is not known whether the macromolecules synthesized after the u.v.- and visible-light treatments were functional. No further clues are offered with regard to how it is possible for 3eV photons to "undo" some of the damage inflicted by 5eV photons.

The authors wish to thank Prof. R. B. Setlow for helpful criticism and advice.

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Received April 1st, 1959

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The presence of carbamyl phosphate synthetase in intestinal mucosa

The distribution of carbamyl phosphate-aspartate transcarbamylase in various mammalian organs indicates that carbamyl aspartic acid is probably synthesized by most tissues[1]. This raises the question of the nature of the carbamyl group donor (or donors) in extrahepatic tissues, and how it (or they) arise. It has been found that carbamyl phosphate synthetase preparations from rat or dog liver are considerably more stable in the presence of Mg^{++} and ATP[2]. A survey of extrahepatic tissues relatively rich in carbamyl phosphate-aspartate transcarbamylase[1] for the presence of carbamyl phosphate synthetase in suitably fortified media was therefore undertaken. The presence of this enzyme in the small intestine is reported in this paper.

Tissues were excised, chilled, cut into small pieces and homogenized at 0° in a glass homogenizer for 1 min with 4 vols. ATP, $MgSO_4$ solution (each 0.01 *M*), pH 7.5. The small intestine (from the dog, rat, and rabbit) was opened and rinsed with cold distilled water. The mucosa and adjacent tissues (including part of the muscular layer) were removed by gentle scraping and then homogenized. Acetone powders of the intestinal preparations were prepared by conventional methods[3]. The acetone powder was extracted with 5 ml/g acetone powder of a solution made up of cold (0°) ATP (0.01 *M*) and $MgSO_4$ (0.01 *M*), pH 7.5, for 15 min, followed by centrifugation at 3600 × *g* for 10 min at 0°. The supernatant solution was used for assay.

Ornithine transcarbamylase was prepared as previously described[2].

Radioactivity of column effluent fractions was determined by counting an aliquot of the fractions as a thin dried film on flat copper disks, using an end-window Geiger-Müller tube.

All assays of enzymic activity were carried out at pH 7.5 at final substrate concentrations as follows: ATP, $1 \cdot 10^{-2}$ *M*; $MgSO_4$, $1 \cdot 10^{-2}$ *M*; L-ornithine, $1 \cdot 10^{-2}$ *M*; NH_4HCO_3, $5 \cdot 10^{-2}$ *M*; acetyl-L-glutamic acid, $1 \cdot 10^{-2}$ *M*; ornithine transcarbamylase, 1 mg protein. The tissue homogenate (0.5 ml) was added to give a final vol. of 1 ml. The incubation was carried out at 38° for 15 min, and was followed by deproteinization with $HClO_4$. Control tubes were deproteinized at zero time. Citrulline was determined according to ARCHIBALD[4].

Of the extra-hepatic rat tissues assayed (small intestine, gastric mucosa, kidney, lung, testis, spleen, caecum), an apparent net synthesis of citrulline was observed only in the case of the small intestine. Since the apparent carbamyl phosphate synthetase activity in the intestinal preparations could have been an artifact introduced by bacterial contamination (carbamate kinase activity), it was necessary to determine whether acetyl-L-glutamic acid was required for optimal citrulline synthesis[5,6]. The results of such an experiment with a rabbit-intestine preparation are shown in Fig. 1.

When homogenates of small intestine or extracts of acetone powder made from intestine were prepared in the absence of ATP and Mg^{++}, essentially no carbamyl phosphate synthetase activity could be detected. Even in the presence of ATP and Mg^{++}, enzyme activity decreased rapidly, and after 15 to 20 min was barely detectable. Because of this instability, it is difficult to compare activity of the small-intestine preparations with liver preparations.

The negative results obtained in this investigation with extrahepatic tissues other

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Abbreviation: ATP, adenosine triphosphate.

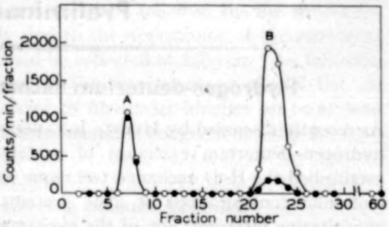

Fig. 1. Citrulline synthesis by extract of acetone-powder preparation of rabbit intestinal mucosa in the presence and absence of acetyl-L-glutamic acid. The complete system contained the following: ATP, 30 μmoles; L-ornithine, 30 μmoles; MgSO$_4$, 30 μmoles; acetyl-L-glutamic acid, 30 μmoles; NH$_4$Cl, 150 μmoles; NaH^{14}CO$_3$, 150 μmoles ($5 \cdot 10^5$ counts/min); D-3-phosphoglyceric acid, 90 μmoles; muscle preparation[7], 6 mg; ornithine transcarbamylase, 4 mg, and acetone-powder extract containing 25 mg protein. Final vol. was 2.7 ml, and the incubation was at 38° for 30 min. The deproteinized incubation mixtures were chromatographed on 1 × 10 cm columns of Dowex-50 H$^+$ (10% cross-linked) 200–400 mesh. Gradient elution was employed. The mixer contained 50 ml water and the reservoir 4.2 *N* HCl. All fractions were 5 ml. ○–○–○ complete system; ●–●–● complete system *minus* acetyl-L-glutamic acid. The identity of peak A was not investigated. Peak B corresponds to the elution position of citrulline.

than small intestine must be interpreted with reservations imposed by the use of a single type of tissue preparation and the limitations of the assay method. Recent studies in this laboratory (R. L. METZENBERG, M. MARSHALL AND P. P. COHEN, unpublished), however, using more sensitive techniques, have confirmed the results presented here. It has been shown previously that preparations of small intestine also contain ornithine transcarbamylase[1]. Whether other enzymes of the urea cycle are present has not been established.

Preparations from rat and dog small intestine gave results similar to those reported for the rabbit. However, it was observed in the case of the dog intestine that the carbamyl phosphate synthetase activity was present not only in the superficial mucosa scrapings, but also in the residual portion of the scraped intestine.

The source of the carbamyl group donor in other extrahepatic tissues remains unanswered. It has been suggested that carbamyl phosphate may arise from citrulline in extrahepatic tissues[1,8]. Attempts to demonstrate this experimentally have been negative[8].

This work was supported in part by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, and the Wisconsin Alumni Research Foundation.

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Received April 9th, 1959

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Preliminary Notes

Hydrogen-deuterium exchange in N-methylacetamide

As recently discussed by HVIDT, JOHANSEN AND LINDERSTRØM-LANG[1,2] work on the hydrogen–deuterium exchange of proteins and peptides dissolved in water has established the H–D exchange technique as a useful tool for the characterization of molecular configurations of some proteins and peptides in aqueous solution. The quantitative interpretation of the exchange results is, however, complicated by the absence of a detailed description of the exchange reaction. In particular no definite quantitative correlation can at present be made between the rates of H–D exchange and the thermodynamic stability in aqueous solution of the folding (secondary structure) of the protein molecules. In order to clarify the H–D exchange mechanism experiments were planned by K. LINDERSTRØM-LANG AND NIELSEN, and in this communication are reported some preliminary date on the H–D exchange of the peptide grouping –CO–NH– of N-methylacetamide. At a later date a discussion will appear on the present exchange results in relation to the H–D exchange of proteins and peptides, together with more complete data.

Rates of the exchange reactions

$$CH_3CONHCH_3 + D_2O \rightarrow CH_3CONDCH_3 + HDO \qquad (A)$$

and

$$CH_3CONDCH_3 + H_2O \rightarrow CH_3CONHCH_3 + HDO \qquad (B)$$

were obtained using a "stopped flow" technique and recording the time course of infrared transmission of the reaction mixture at fixed wavelengths in the infrared transmission "windows" of H_2O and D_2O. A 0.1-ml all-glass syringe (I) with precision bore was filled from an aged mixture of 260 µl D_2O (or H_2O) and 0.715 g N-methylacetamide. Likewise a 0.5-ml syringe (II) was filled from a mixture of 650 µl H_2O (or D_2O) and of 10 µl of a 1.2 *M* citrate or acetate buffer solution made up with H_2O and KCl to adjust the ionic strength to approximately 1.0. The syringes I and II were driven by a motor arrangement carefully constructed to give a delivery ratio of 1/80 (I/II) at a rate of approximately 0.25 ml total volume per sec. Via two hypodermic needles and a mixing chamber the solutions were mixed and forced into a 0.10-mm optical cell with fluorite windows placed in the beam of a Perkin Elmer model 13U Infrared Spectrometer arranged for single-beam operation with a fluorite prism. Thermospacers kept the cell temperature at 22°. pH of reaction mixture B (approximately 99.7 vol. % H_2O, *i.e.* 0.997 vol. H_2O/vol. water) was determined with an ordinary glass electrode-saturated KCl calomel electrode system, whereas "pD" of reaction mixture A (approximately 98.0 vol. % D_2O) was determined with an electrode system consisting of a glass electrode and a calomel electrode made up with a saturated solution of KCl in water containing 98 vol. % D_2O and standardized against HCl–KCl solutions in 98.0 vol. % D_2O[3]. Reaction A was followed by the increasing transmission at 1580 cm^{-1} (essentially the disappearance of the amide II band of the protonated amide), but it could also be followed at 1620 cm^{-1} and at 1650 cm^{-1}, points on the slopes of the amide I band at which transmission changes occur due to a displacement of the latter band following deuteration. Rate data obtained at these wavenumbers agreed within experimental accuracy. Reaction B was followed by the decreasing transmission at 2600 cm^{-1} (predominantly due to the appearance of the stretching mode ν(D–OH)) but corresponding data could be collected at 2400 cm^{-1} by following the increasing transmission (primarily due to the disappearance of ν(N–D)). As determined for A and B the rates correspond to first-order kinetics up to at least 70 % conversion. The calculated first-order rate constants are given in Table I.

TABLE I

FIRST-ORDER RATE CONSTANTS OF H–D EXCHANGE REACTIONS

A		B	
pD	$\frac{k_A}{min^{-1}}$	*pH*	$\frac{k_B}{min^{-1}}$
3.23	23	3.41	7.7
3.88	6.4	4.19	1.6
4.62	1.3	4.87	0.48
5.27	0.41	5.57	0.71
5.97	0.77		

Reactions A and B proceed with minimal rates around pD 5.4 and pH 5.1 respectively. The points in Table I on the acid side of these acidities indicate that in both reactions we are dealing with first-order acid catalysis with rate constants of the order of $k'_A = k_A/a_D \approx 5 \cdot 10^4$ l/mole/min and $k'_B = k'_B = k_B/a_H \approx 2.5 \cdot 10^4$ l/mole/min. a_H and a_D are hydrogen and deuterium ion activities. These values agree well with the value $(2.3 \pm 0.2) \cdot 10^4$ l/mole/min of the rate constant for the exchange of the proton in N-methylacetamide dissolved in HCl–H_2O mixtures, reported recently[4] on the basis of nuclear magnetic resonance studies at 23 ± 2°. The observed isotope effect $k'_A/k'_B = 2.0$ is in apparent agreement with a reaction mechanism involving addition of one hydrogen ion to the amide prior to the rate-determining step[5].

It is a pleasure to acknowledge the helpful comments of Professors M. OTTESEN and R. LUMRY and the technical assistance of Mrs. KIRSTEN MIKKELSEN.

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Received September 30th, 1959

A method for estimating changes in bound water associated with myocardial contraction

In connection with a study of the reactions of dried muscle proteins with water[1], a method has been developed for estimating changes in the binding of water to proteins that could be applied to both solutions and tissues. The possibility of detecting bound water by the use of radio-frequency spectroscopy was suggested by FREYMANN's observation[2] that centimeter wavelengths are not absorbed by free water but are absorbed by bound water below 0°. Unfortunately most of the existing methods for measuring the absorption of radio frequency energy require large samples with exactly known dimensions which can only be cooled very slowly. Since a rapid and reversible reaction was being studied, it was necessary to develop methods which could be applied to small irregularly shaped samples that could be frozen rapidly to fix them in the desired physiological state. The method which has been developed is based upon measuring the temperature change resulting from the conversion of the absorbed electromagnetic energy into heat. Methods based on the same principle have been employed by DEBYE[3] and more recently by OCLEY, FERRY AND SHACK[4] in studying the dielectric constants of protein solutions where it was found to give good agreement with the results of other methods.

The apparatus consists of a oscillator operating at 1,000 Mc and a tuned line which includes a detachable copper-constantan thermocouple at its end. The line is tuned so that there is maximum power at the thermocouple, and the tuning and power level are not changed during a series of determinations. The temperature of the sample, which is frozen on the thermocouple, is controlled by adjusting the flow of room-temperature N_2 entering a Dewar flask cooled with liquid nitrogen. The temperature of the sample was stabilized before each reading and then the high-frequency power turned on for 5 sec to 1 min depending on the heating rate. The heating, as recorded by a galvanometer, is converted into the rate of temperature change, in °/min and plotted as a function of the temperature of the sample at the moment that the power was turned on. In all cases, the heating rate of pure ice, which is at a constant level of 1°, is subtracted from the observed experimental values.

In general most of the absorption by frozen solutions occurs above the eutectic temperature of the system and the absorption at the lower temperatures is roughly proportional to the solutes ability to "bind" water or inhibit its complete crystallization. 0.1 *M* NaCl, which absorbs maximally at −18°, has no absorption below −25° and thus does not obscure the absorption of protein solutions or tissues. Glycine solutions (Fig. 1) absorb most strongly at the pHs of maximum buffering activity with little absorption at the intermediate pHs. The same effect is seen in solutions of acetic acid and ammonium hydroxide—maximum absorption is associated with pH of maximum buffering activity and these maxima occur at approximately the same temperatures as those seen in glycine. (The curves obtained from albumin solutions[5] are considerably more complicated, but they show the same tendency to shift towards the lower temperatures as the pH is raised.) The curves derived from four frog ventricles are shown in Fig. 2. Each ventricle weighed about 160 mg. It is tempting to interpret the difference seen between the contracted and relaxed ventricles in terms of changes in the binding of water occurring during contraction. However, at present, other explanations for the observed differences cannot be excluded—the possibility exists that part of this difference is due to the fact that the two pairs of ventricles were under different degrees of stress at the time of freezing.

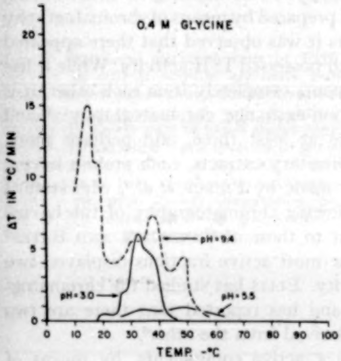

Fig. 1. Effects of pH on the absorption of 0.4 *M* glycine. The peak above −20° is due to the sodium of the NaOH which was used in adjusting the pH. Readings were taken every 2° as the temperature was raised. The solutions were held on the thermocouple by hanging over it a strip of glass-fiber filter paper which was then dipped into the solution, withdrawn and frozen in liquid nitrogen.

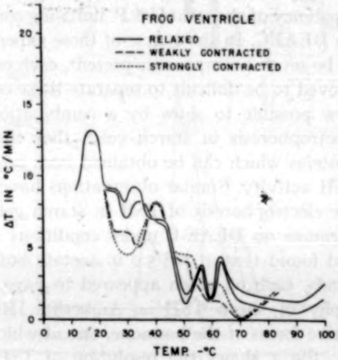

Fig. 2. Absorption by frozen frog ventricles. The thermocouple was placed within the ventricle which was then placed in Ringer's solution at 2° to reduce the rate of contraction. Frozen in isopentane at −170°. After 5 min in liquid N_2, the temperature of the sample was raised to −100° where the first readings were made.

With the limitations of the present method, it is not possible to state definitely what type of absorption is occurring. According to FREYMANN[6], it is probably due to induced polarization of bound water. Although there is good evidence that electromagnetic energy at these wavelengths is absorbed by bound water in other solid-state systems, it must be pointed out that the data presented here do not eliminate other possibilities in frozen biological material.

This work was supported by grants from the Cleveland Area Heart Society and the American Medical Association and was greatly facilitated by the generous loan of some of the necessary equipment from the Bird Electronic Corporation of Cleveland, Ohio.

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Received September 3rd, 1959

On the existence of multiple forms of bovine thyrotrophin

In 1957 CONDLIFFE AND BATES[1] reported that thyroid-stimulating hormone having a potency of about 20 U.S.P. units/mg could be prepared by means of chromatography on DEAEC. In the course of these experiments it was observed that there appeared to be several components present, each of which possessed TSH activity. While it has proved to be difficult to separate these components completely from each other, it is now possible to show by a combination of ion-exchange chromatography[1-3] and electrophoresis in starch gels[4], that there are at least three, and perhaps more, proteins which can be obtained from bovine pituitary extracts, each protein having TSH activity. Similar observations have been made by PIERCE *et al.*[5], who studied the electrophoresis of TSH in starch gels, following chromatography of the bovine hormone on DEAE-C under conditions similar to those of CONDLIFFE AND BATES[1] and found that at pH 5.0 in acetate buffer the most active fractions displayed two bands, each of which appeared to have activity. ELLIS has studied the chromatography of sheep TSH on Amberlite IRC-50 and has reported that there are two active forms of the hormone, one of which is derived from the other[6].

Fig. 1 shows the resolution of TSH into 2 active components, by means of chromatography on DEAEC at pH 9.5. The two peaks of activity were recovered and the pooled fractions (B3 and B4, Fig. 1) were found to have potencies of 17 and 13 units/mg respectively. After electrophoresis in starch gel, fraction B3 displayed 2 bands and B4 3 bands as shown in the upper part of Fig. 2. When the major portion of the same gel was cut into 2-mm strips, extracted, and bioassayed[7], several active components were found, as shown in the lower part of Fig. 2. Peaks of activity corresponding to bands 1 and 2 were obtained from fraction B3. An additional peak of biological activity at 15 mm was found in B3, for which no band was observed. In the case of fraction B4, peaks of activity were found corresponding to bands 2, 3 and 4. Band 1 was not seen in fraction B4, although a small amount of TSH activity was found at 25 mm in this fraction. The principal active component of fraction B4 corresponded to band 3, which was missing in the pattern from fraction B3, although

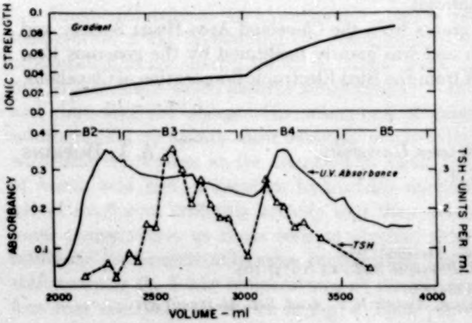

Fig. 1. Re-chromatography of TSH at pH 9.5 in sodium glycine buffer on DEAEC. Column size: 1.9 × 15 cm, temp. 2°, fraction size 10 ml. Solid line, bottom part of figure, u.v. absorption of the effluent at 276 mμ; Δ TSH concentration in U.S.P. units/ml; solid line in upper part of figure shows change in ionic strength of eluting solvent. Fractions pooled as indicated by brackets. 532 mg TSH (5 units/mg) placed on column in 6 ml buffer.

Abbreviations: TSH, thyroid-stimulating hormone; MSH, melanocyte-stimulating hormone; DEAEC, diethylaminoethylcellulose.

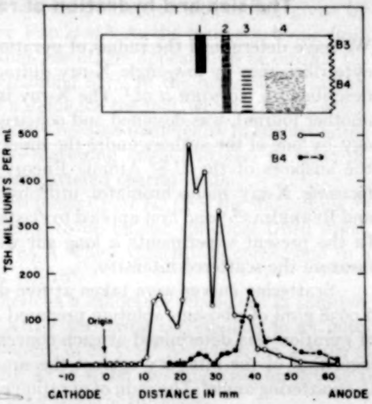

Fig. 2. Starch-gel electrophoresis of TSH at pH 9.5 in 0.01 *M* sodium glycinate buffer. Upper part of figure shows bands revealed by staining a portion of gel with naphthalene black 12B. Lower part of figure shows concentration of TSH when 2-mm sections of gel were extracted with 2 ml 0.1 *M* $NaHCO_3$. Duration of run was 16 h at 150 V, 15 mA, trough size 40 × 5 × 300 mm. ○—○ preparation B3 in Fig. 1 (15 units); ●---● preparation B4 Fig. 1 (7 units).

a small amount of TSH activity was found at 39 mm. Thus the principal active components of fractions B3 and B4 are found at 25 and at 39 mm respectively, after electrophoresis and are clearly different.

Whether these components are artifacts in the sense that they arise from a single molecular species of TSH by some chemical alteration during isolation, or whether they exist as distinct forms of the hormone in nature is a question to be answered by further experimental work. Instances are known where the same hormonal activity may reside in different molecules, as exemplified by α- and β-MSH[8]. However, it has been shown by SHEPHERD *et al.*[9] that β-corticotrophin can be deamidated without loss of potency under mild conditions not unlike those employed in the chromatography of TSH on DEAEC at pH 9.5. If different forms of TSH exist in nature, the genetic aspects of the problem will be of interest, since all pituitary extracts are drawn from pools of thousands of glands of mixed genetic origin.

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Received August 12th, 1959

The size and hydration of rabbit-reticulocyte ribosomes

We have determined the radius of gyration and other parameters of rabbit-reticulocyte ribosomes by low-angle X-ray diffraction, using the methods of interpretation described by Guinier *et al.*[1]. The X-ray instrumentation, which will be described in another journal, was designed and constructed at the California Institute of Technology by one of the authors under the supervision of Dr. J. W. M. DuMond and under the auspices of the U.S. Atomic Energy Commission. The instrument is a point-focusing X-ray monochromator utilizing the geometry developed by Despujols[2] and Berreman[3,4] and first applied to elastically bent quartz crystals by Berreman[3,4]. In the present experiments a long slit was used in front of the Geiger counter to measure the scattered intensity.

Scattering curves were taken at five different concentrations between 0.113 and 0.0038 g/ml of ribosome solution prepared as described by Dintzis *et al.*[5]. The radius of gyration was determined at each concentration by means of the usual plot of the logarithm of the intensity against the square of h, where $h = 4\pi/\lambda \sin ø/2$ and ø is the scattering angle[6]. The radii of gyration obtained at each concentration were plotted against concentration, and extrapolated to zero concentration. A slit-width correction was made in the Gaussian region of the scattering curve by approximating the central unscattered beam with a Gaussian curve also, and this raised the radius of gyration by about 1 % to a value of 108 Å, which should be accurate to about 2 %, according to our estimate of errors. This is probably about 2.5 % high due to the presence of about 9 % of the sample in the form of larger particles[5]. The presence of another 9 % as smaller particles should not appreciably affect the radius of gyration.

We searched for but did not find any evidence of the secondary scattering maxima which would be present for spheres of uniform density, and therefore conclude that the "uniform hard sphere" model does not fit this particle.

An estimate was made of the mol. wt. of the particles by means of the formula $I(o) = I_e(o)\bar{N}n^2$ given by Guinier *et al.*[7]. This required the electron density of the

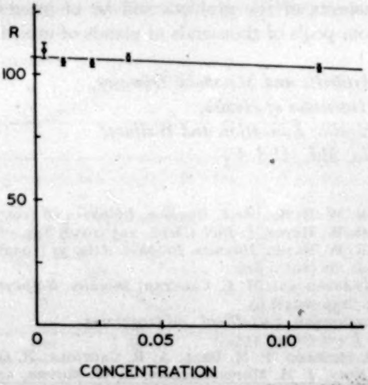

Fig. 1. Plot of R, the apparent radius of gyration (Å) versus the concentration of the ribosome solution (g/ml). R is determined by a plot of log I versus the square of h at each concentration. Estimated errors are indicated and are mainly due to counting errors.

ribosomes, which was calculated from the specific volume of 0.63 ml/mg given by DINTZIS *et al.*[5] and the composition given by T'SO *et al.*[8]. As the main scattering from the 9 % of the particles which were larger than the others appeared to be outside the region of accurate experiment, only the remaining 91 % of the mass of the sample was used in the computation. We obtained a value for the mol. wt. of $4.0 \cdot 10^6$, which should not be trusted to better than 20 %, mainly because of difficulty in extrapolating the value of the scattered intensity to zero scattering angle and also to some inaccuracy in measuring the intensity of the unscattered central beam.

Electron-microscope pictures taken by Professor CECIL HALL of the Massachusetts Institute of Technology showed the ribosomes to be almost spherical when dried, with a diameter of 200 Å and a height of 185 Å. These dimensions correspond to a dry volume of $3.9 \cdot 10^6$ Å^3 and a mol. wt. of $3.7 \cdot 10^6$.

If one takes a uniformly hydrated sphere as a model for ribosomes in solution, then the radius of gyration measured by X-ray scattering corresponds to an outer radius of 170 Å in solution, or a hydrated volume of $20.5 \cdot 10^6$ Å^3, which dries down to a volume of $3.9 \cdot 10^6$ Å^3. The loss of $16.6 \cdot 10^6$ Å^3 of water per ribosome on drying is equivalent to saying that the hydration is 2.7 g water/g ribosome.

These conclusions are surprisingly similar to those based on sedimentation, viscosity and partial specific volume[5], which indicated a mol. wt. of $4.1 \cdot 10^6$ and a hydration of 2.6 g water/g of anhydrous particle, and also to those based on light scattering[5], which showed a mol. wt. of $4.0 \cdot 10^6$ and an almost spherical shape in solution.

All the data taken together lead to the picture that the rabbit reticulocyte ribosome is an almost spherical sponge-like structure of mol. wt. $4.0 \cdot 10^6$ (anhydrous), containing approximately 80 % of its enclosed volume as water of hydration.

The authors wish to thank MR. H. YURA for assistance in taking X-ray data.

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Received August 29th, 1959

Use of chloramphenicol in the study of nitrogen fixation

Molecular N_2 fixed by biological agents is thought to be assimilated as NH_3 by reductive amination of α-ketoglutarate to form glutamate, followed by transamination to form other amino acids[1]. However, this sequence is not supported[2, 3] by the distribution of ^{15}N in ammonia and glutamate isolated from growing cultures of *Azotobacter vinelandii* after 5-min exposure to $^{15}N_2$. Glutamate had the higher level of isotope which suggests that a compound other than the NH_3 is precursor since "steady state" fixation demands that the specific activity of the precursor be greater than that of the product[4].

We seek to resolve this apparent contradiction by the use of metabolically blocked preparations of *Azotobacter*. We have found that inhibition of protein synthesis by chloramphenicol promotes accumulation of the reaction intermediates of nitrogen assimilation, including ammonia and acid-soluble components.

Log-phase cultures of *Azotobacter vinelandii O* grown in BURK's medium[5] with 2 % sucrose were incubated with 100 μg chloramphenicol/ml for 30 min and then exposed to gas containing 95 % $^{15}N_2$, O_2 and He (2 : 2 : 6)[6]. The population increase of the culture was essentially arrested at this concentration of antibiotic, O_2 uptake was inhibited about 40 %, but the rate of fixation of the ^{15}N was reduced only 20 % compared to controls. Thus, the rate of fixation of nitrogen does not appear to depend upon cell proliferation.

Table I shows that chloramphenicol suppressed the incorporation of ^{15}N into protein and increased the isotope content of the acid-soluble, nonprotein fraction. The ratio of acid-soluble to protein residue ^{15}N was increased 10-fold. Uptake of the isotope in the protein fraction was reduced to about one-fifth that of the untreated controls. Some of the ^{15}N incorporation into the protein residue can probably be attributed to amidation of free carboxyl groups of cell protein[2].

TABLE I

EFFECT OF CHLORAMPHENICOL ON FIXATION AND ASSIMILATION OF NITROGEN

Aliquots of a suspension of *Azotobacter* cells ($4 \cdot 10^8$ cells/ml) in Burk's medium with 0.2 % sucrose were preincubated with the indicated amounts of chloramphenicol for 60 min at 30° while the untreated control was kept at 0°. Samples were exposed to the $^{15}N_2$ gas mixtures for 30 min. H_2SO_4 (0.1 *N* final concn.) was used as protein precipitant.

Chloramphenicol (μg/ml)	*^{15}N incorporation*		
	A acid-soluble (atom-% ^{15}N excess)	*B Protein residue (atom-% ^{15}N excess)*	*Ratio $A/B \times 100$*
none	0.563	3.027	18.8
200	1.178	1.018	115.8
400	1.176	0.634	185.5

Table II shows the effect of chloramphenicol on the distribution of nitrogen from fixation. After 3-h incubation the acid-soluble fraction of the treated cultures contained about 35 % of the nitrogen of the culture; the same fraction of the control cultures contained about 15 % of the total nitrogen. Although ammonia-N and acid-soluble N were higher in the chloramphenicol-treated preparations, the total N of the treated samples was only about 65 % of the control. Thus nitrogen fixation is suppressed markedly at 400 μg chloramphenicol/ml.

TABLE II

EFFECT OF CHLORAMPHENICOL ON THE DISTRIBUTION OF NITROGEN FROM FIXATION

The culture consisted of 50 ml of a suspension of *Azotobacter* cells ($4 \cdot 10^8$ cells/ml) in BURK's medium with 0.2 % sucrose which was incubated for 3 h in an incubator shaker at 30° with or without 400 µg chloramphenicol/ml. Cell protein was precipitated from the culture with H_2SO_4 (0.1 *N* final concn.) and ammonia was distilled from the supernatant solution at room temperature. Nitrogen was determined colorimetrically after nesslerization.

Expt.	*Chloramphenicol (µg/ml)*	*Nitrogen of Culture*			
		Ammonia N (µg)	*Acid-soluble N (µg)*	*Protein N (µg)*	*Total N (µg)*
1	400	22	1050*	2160	3232
	none	16	767	4283	5066
2	400	19	1250*	2000	3269
	none	11	1000	4183	5194

* Values are corrected for chloramphenicol-N.

We are now isolating and identifying the intermediates of the fixation and assimilation processes which have accumulated in the acid-soluble fraction. The results of this investigation will be published subsequently.

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Received October 23rd, 1959

Biochim. Biophys. Acta, 37 (1960) 154–155

Deoxyribonucleic acid synthesis and ultraviolet-induced mutation

The exposure of bacteria to ultraviolet light results in the production of potential mutants. The frequency of mutants can be markedly reduced by post-ultraviolet treatments such as exposure to visible light[1] and growth in a medium containing CMP[2]. It has been suggested[3] that irradiation produces premutational damage (mutagens) that may be lost spontaneously or lead to mutation at the time of a "terminal event". Assuming that the production of unstable mutagens is instantaneous, mutagenesis could be reduced by increasing the rate of loss of mutagens, or by delaying the "event" during which mutagens produce mutations. Data presented below suggests that CMP reduces mutation frequency by delaying DNA synthesis.

A culture of *Escherichia coli* B/r requiring tryptophan for growth was grown to

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; CMP, chloramphenicol.

stationary phase in Difco nutrient broth. Washed bacteria were irradiated in 0.9 % saline with a General Electric germicidal lamp with a dose allowing 70 % survival. The bacteria were diluted in the both give a concentration of $2 \cdot 10^8$ bacteria/ml and aerated at 37°. At each sampling time, two aliquots were removed. One was chilled, washed and assayed immediately. CMP was added to the second aliquot to give a concentration of 10 μg/ml, and after an additional hour at 37° the bacteria were washed and assayed. The concentrations of tr^+ and tr^- bacteria were assayed on plates containing a glucose-salts medium supplemented with 5 % nutrient broth. This medium permits all tr^+ mutants which are phenotypically expressed after 5–6 cell divisions to form colonies.

In Fig. 1, the left ordinate indicates the relative amount of protein[4], DNA[5], and RNA[6], and the right ordinate indicates mutant frequency after 1 h of CMP "challenge"[7]. The solid lines in Fig. 1a show the increase in each bacterial constituent during the growth of the irradiated culture in nutrient broth. Fig 1b presents data obtained in the same experiment with the exception that the assays were made after CMP challenge, so that the first point is plotted at 60 min. Superimposed on the curves in Fig. 1a and 1b is a dashed line indicating the mutant frequency determined after CMP challenge. It should be emphasized that the time axis in Fig. 1a indicates the time that CMP was added.

The mutant frequency curve in Fig. 1a parallels the RNA curve and shows no obvious relationship to the curves for DNA and protein. Comparisons of this type prompted DOUDNEY AND HAAS[7,8] to suggest that RNA synthesis causes "mutation fixation". However, comparison of 1a and 1b shows that considerable DNA (and RNA) synthesis occurs in the presence of CMP when it is added after the irradiated culture has grown in both for a short time[9,10]. It thus appears more pertinent to compare mutant frequency after CMP with macromolecular concentrations assayed at the same time. In Fig. 1b, the frequency of mutants increases to a maximum as the DNA content of the culture increases 140 %. This supports a previous suggestion[3] that the "terminal event" is DNA synthesis. Since several doublings of DNA content

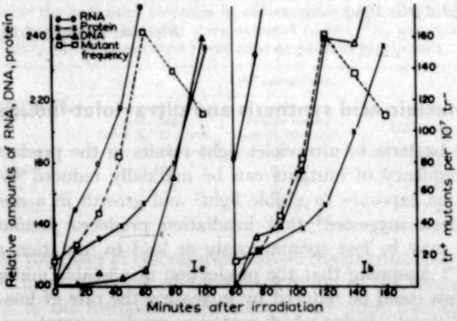

Fig. 1.

occur in all aliquots after plating, it is obvious that DNA synthesis after a certain critical time does not increase mutation above a minimal frequency. This suggests that mutagen concentration reaches a minimum value. Additional experiments indicate that after low doses of ultraviolet the critical time is about 100–120 min. When bacteria irradiated with low doses are grown in broth, DNA doubling usually occurs in 100 min. The decrease in mutant frequency after the first doubling of DNA is correlated with cell division and indicates the segregation of mutant and non-mutant bacteria.

Table I presents data from an experiment in which aliquots of an irradiated culture growing in broth were removed at intervals and subjected to both CMP and saline challenge. Although RNA had almost doubled after 15 min, and more than doubled after 30 min, saline challenge reduced the mutant frequency to a minimal value. CMP added at 30 min was ineffective in reducing mutant frequency; this was expected since DNA had doubled at the time of assay after CMP. Experiments in which bacteria were challenged after longer incubation in broth showed that saline challenge did not reduce mutant frequency after the amount of DNA had doubled.

TABLE I

Time (min) in nutrient broth	*Relative amounts*			$tr^- \times 10^7$ *per ml*	$tr^+ \times 10^{-4}$ *per ml*	tr^+ *per* 10^7 tr^-
	RNA	*Protein*	*DNA*			
0	100	100	100	150	12.3	82.0
+ 60 min CMP	153	107	107	138	2.2	16.2
+ 60 min sal.	90	100	97	110	1.4	12.4
15	194	114	99	125	10.4	83.0
+ 60 min CMP	201*	92	105*	86*	2.6	30.5
+ 60 min sal.	97	86	86	145	1.7	10.7
30	220	127	115	181	17.1	94.1
+ 60 min CMP	210*	118	182*	102*	9.9	97.0
+ 60 min sal.	188	106	112	130	1.6	12.6

CMP = Nutrient broth + 10 μg chloramphenicol/ml.
Sal. = 0.9 % NaCl + 0.2 % glucose.
* Chloramphenicol treatment led to appreciable killing of bacteria not in the stationary phase. Relative amounts of DNA and RNA were not corrected to account for killing. Such a correction would almost certainly increase the figures listed, but the level of NA synthesis in "dead" cells in not known at this time.

This work has been supported by a grant from the National Science Foundation.

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Received September 28th, 1959

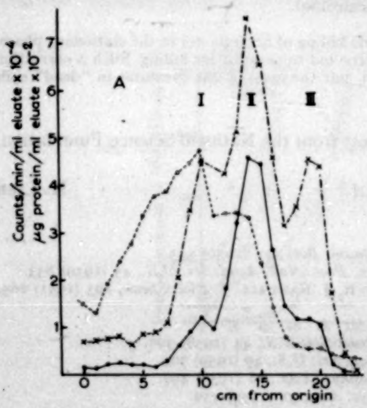
The synthesis of RNA and protein in the cells of *Escherichia coli* irradiated with ultraviolet light as revealed by electrophoresis on starch column

It has been reported by DOUDNEY AND HAAS[1-3] that the mutation of *Escherichia coli* induced by u.v. irradiation seems to result from a modification of the RNA macromolecules synthesized after irradiation. The correlation between mutation frequency and protein synthesis was shown simultaneously by these authors[2,3] and by WITKIN[4]. We have observed the metabolic heterogeneity of RNA in irradiated *Escherichia coli* B(H)[5]. In order to obtain further information on the effects of irradiation, it seemed to be of interest to study whether or not any modification occurred in the distribution of RNA and protein macromolecules synthesized after irradiation. When sonicates of the cells labeled with ^{32}P or ^{35}S during postirradiation incubation were fractionated by electrophoresis on starch column we obtained atypical patterns with respect to nucleic acid and protein. Some preliminary results will be presented in this report.

The bacterial strain used was *Escherichia coli* B(H). The Tris–glucose medium[6] was used. Sonicates were prepared by the use of a 20-kc sonic oscillator for 7 min. The length of the column was 40 cm. The runs were made with a potential gradient of 11 V/cm in a cold room. Starch was mixed with Tris buffer, pH 7.8, I 0.02. The procedure for u.v. irradiation was the same as reported previously[5]. The treatments and analytical methods after each run were similar to those reported by PARDEE *et al.*[7].

Different types of experiments with respect to incubation time and isotope were performed. The irradiated cells (survivals were 10^{-5}) were incubated for either 10 min (Fig. 1) or 90 min with ^{32}P, washed, and disintegrated. In other experiments, ^{35}S was substituted for ^{32}P. In each experiment control cells which did not receive u.v. irradiation were treated in paralell. Typical results are shown in Figs. 1 and 2.

It can be seen from these results that during a relatively short period (10 min) of post-irradiation incubation, the synthesis of the RNA fraction which corresponds



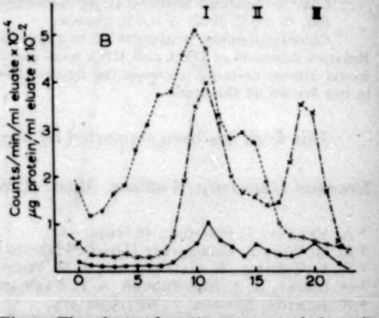


Fig. 1. The electrophoretic patterns of the cell components of normal (A) and u.v.-irradiated (B) *Escherichia coli* B (H)– Post-irradiation incubation was continued for 10 min. Protein (– – ○ – – total ^{32}P (–·–×–·–), [^{32}P]RNA (—●—).

Abbreviations: RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

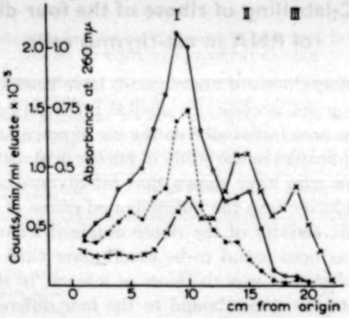


Fig. 2. The electrophoretic patterns of the proteins of normal (—O—) and u.v.-irradiated (--●--) *E. coli* B (H). As a comparison the pattern for the absorption at 260 mμ (irradiated) is also shown (—×—). Post-irradiation incubation was continued for 90 min.

to one of the three peaks (peak II in Fig. 1) was inhibited considerably. This was also observed with respect to the pattern of total ^{32}P. When the incubation period was prolonged to 90 min, the peak II which failed to appear after 10-min incubation was apparently restored. As shown in Fig. 2, no significant difference could be observed in the ^{35}S-labeled protein pattern between irradiated and non-irradiated cells. However, a slight inhibition of the incorporation of ^{35}S into the protein of peak II can be seen in irradiated cells. This peak resides just at the position corresponding to the peak II of [^{32}P]RNA or of total ^{32}P mentioned above.

According to the results obtained by DOUDNEY AND HAAS[3], mutation frequency decreased when 5-hydroxyuridine was added 30 min after post-irradiation incubation but not when it was added earlier. Comparing this to the results presented in this report it seems probable that the RNA, the synthesis of which was inhibited by 5-hydroxyuridine, corresponds to the fraction of peak II which disappears from the normal pattern after at least 10 min post-irradiation incubation. We are at present studying whether or not this RNA fraction corresponds to the unstable RNA mentioned in our preliminary report[5].

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Received August 17th, 1959

Comparison of ^{14}C-labelling of ribose of the four different nucleotides of RNA in rat-thymus cells

Many aspects of the biosynthesis of nucleic acids have been studied in tracer experiments using ^{32}P and various isotopically-labelled precursors of the purine and pyrimidine bases. Work has been initiated[1,2] on the use of precursors of ribose and deoxyribose as radioactive indicators in the study of nucleic acid metabolism in mammalian tissues. Experiments *in vitro* have shown that rat-thymus cells readily incorporate the isotope from [^{14}C]glucose into the purine-bound ribose of their RNA[1]. In similar experiments the specific activity of the ribose obtained from the mixed pyrimidine nucleotides of RNA has been found to be much lower than that derived from the mixed purine nucleotides[2,3]. It was therefore of interest to study the incorporation of ^{14}C from glucose into the ribose bound to the four different bases of RNA. The results reported in this communication show striking differences in the specific activities of these ribose moieties.

The thymus glands from male albino rats weighing 130–150 g were collected; a suspension of the thymus cells was prepared[4], and the cells were incubated in the presence of [^{14}C]glucose. At the end of the incubation the acid-soluble material was extracted with 0.6 *N* $HClO_4$, and the lipids by refluxing with chloroform–methanol (1 : 1). In Expt. 1 the residue was fractionated in 1 *N* KOH at room temperature according to the method of SCHMIDT AND THANNHAUSER[5]. Paper chromatography of alkaline hydrolysate of RNA was carried out in isopropanol ammonia[6], and the ribonucleotides were further purified by paper electrophoresis[7]. In Expt. 2 the nucleic acids were extracted with 10 % NaCl at 100° and the sodium nucleates were then hydrolyzed in 0.3 *N* KOH[8]. The nucleotides derived from RNA were then separated by paper electrophoresis[7]. Ribose was liberated from adenylic and guanylic acids by acid hydrolysis, and from cytidylic and uridylic acids by bromination with subsequent hydrolysis[9]. It was then purified by paper chromatography using ethyl acetate–acetic acid–water as solvent[10]. Aliquots of the ribose eluates were taken for determination with orcinol[11], and for counting.

The results (Table I) confirm the earlier observations that, under experimental conditions similar to those described here, the incorporation of ^{14}C into the pyrimidine ribose is lower than that into the purine ribose. Moreover, they show that there is a great difference in the degree of labelling of the ribose attached to the two different pyrimidine bases, and that the specific activity of the cytidylic acid ribose is the lowest of the four ribose values.

Whether the ribose moieties of all four types of nucleotide which form the RNA molecule arise from the same precursor is a question which cannot yet be answered definitely. However, in view of the work indicating the role of 5-phosphoribosyl pyrophosphate in the biosynthesis of the purine and pyrimidine nucleotides[13,14], it could be assumed that these moieties have their origin in a common pool of ribose (or its phosphorylated products). It could thus be inferred that the ribose of cytidylic acid is renewed *in vitro* to a much lesser extent than the ribose moieties of the other nucleotides. This could be interpreted as arising from differences in the rate of renewal of the four ribonucleotides, possibly through corresponding differences in the rate

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Abbreviation: RNA, ribonucleic acid.

TABLE I

INCORPORATION *in vitro* OF ^{14}C FROM [^{14}C]GLUCOSE INTO THE RIBOSE OF RNA OF RAT-THYMUS CELLS

Each incubation vessel contained 6 ml of cell suspension, obtained from 8 thymus glands, in oxygenated Krebs-Ringer phosphate solution[12] containing 5 mg/ml of generally labelled [^{14}C]-glucose* ($4.62 \cdot 10^4$ counts/min/µmole in Expt. 1 and $4.84 \cdot 10^4$ counts/min/µmole in Expt. 2). The vessels were shaken at 37° for 3 h, the evolved CO_2 being absorbed in KOH. In each experiment the contents of two vessels were pooled at the end of the incubation.

Source of ribose	*Specific activity (counts/min/µmole)*	*Relative ^{14}C incorporation*
Expt. 1		
Adenylic acid	590	100
Guanylic acid	530	90
Cytidylic acid	36	6
Uridylic acid	156	26
Expt. 2		
Adenylic acid	585	100
Guanylic acid	446	76
Cytidylic acid	16	3
Uridylic acid	135	23

* Generally labelled [^{14}C]glucose was obtained from The Radiochemical Centre, Amersham.

of breakdown and re-synthesis of the N-glycosidic linkage between the different bases and ribose.

I wish to thank Prof. J. S. MITCHELL, F.R.S., for his interest and Dr. B. E. HOLMES for her encouragement and helpful discussion.

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Received October 24th, 1959

The sedimentation properties of native and denatured deoxyribonucleic acid

In a recent report[1] on the thermal denaturation of DNA it was shown that thermally denatured and native DNA had similar sedimentation coefficients in 0.2 *M* NaCl. Mention was also made of the apparent conflict of this result with that yielded by viscosity and light-scattering measurements. Thus, the apparent similarity of the two states of DNA which follows from a study of sedimentation in 0.2 *M* NaCl is not consistent with the present ideas concerning the denatured state, *i.e.*, a collapsed molecule with similar molecular weight to native DNA.

Investigations made by the present authors, and to be reported in full later, may throw some light on this problem. A study of salt-free DNA solutions[2] has shown that denaturation occurs, on dilution of a concentrated solution, over a critical zone of concentration extending from $50–4 \cdot 10^{-5}$ *M* DNA. At concentrations above this region solutions consist essentially of native DNA while dilution below this zone results in denaturation. Apparently within the critical zone the two forms co-exist.

If a salt-free solution is denatured by dilution and then reconcentrated by freeze drying, the sample exhibits (in 0.2 *M* NaCl) a similar sedimentation coefficient and concentration dependence of the coefficient to that of the native sample. It necessarily follows that a mixture of denatured and native molecules, in 0.2 *M* NaCl, cannot be resolved in the ultracentrifuge. Although denaturation has been produced by a different means in the present case, the result is in agreement with that found for thermally denatured samples[1].

When a salt-free solution is diluted to $2 \cdot 10^{-4}$ *M* DNA we expect, from results to be published, to have a mixture of native and denatured molecules. Sedimentation analysis of this salt-free solution again shows that no resolution into two distinct sedimenting species is possible. However, the boundaries obtained in such an experiment, Fig. 1(h), do show a diffuse leading edge in addition to a hypersharp trailing side. If this solution is now made 0.2 *M* with respect to NaCl (keeping the DNA concentration at $2 \cdot 10^{-4}$ *M*), one boundary only results although the denatured material is still present.

If a $2 \cdot 10^{-4}$ *M* DNA solution is examined at certain NaCl concentrations lower

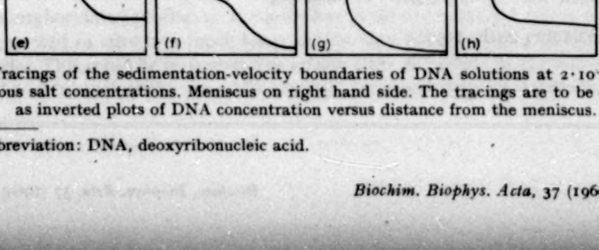

Fig. 1. Tracings of the sedimentation-velocity boundaries of DNA solutions at $2 \cdot 10^{-4}$ *M* DNA and various salt concentrations. Meniscus on right hand side. The tracings are to be considered as inverted plots of DNA concentration versus distance from the meniscus.

Abbreviation: DNA, deoxyribonucleic acid.

than 0.2 M separation into two boundaries has been found to occur. Fig. 1 shows the sets of boundaries formed for a series of solutions at decreasing salt concentrations. It can be seen that the two species have different sedimentation properties at salt concentrations between 50 and $1.0 \cdot 10^{-4}$ M NaCl. Experiments on completely denatured material, prepared by dilution and freeze drying, show that the slower component is to be identified with denatured DNA. These observations show that low salt concentration is one factor at least, which allows the expected differences between native and denatured DNA to be experimentally observed. It is unfortunate, but inevitable, that the conditions needed for resolution of these two species are also those which will produce some denaturation of the native component.

The explanation of this phenomenon must await further investigations due to the rather complex processes that will accompany the separation of the two boundaries. It has been found, for example, that denaturation by dilution occurs over a critical concentration range within which both native and denatured species coexist. Because of this, any decrease in the concentration will cause further native molecules to denature. As the solution within and behind the leading boundary (which is to be associated with native DNA) is necessarily at a lower concentration than the bulk solution, a change in the proportions of denatured and native species must take place within part of the boundary. Consequent changes in conditions may possibly explain the unexpected boundary spreading of the native species. Further complications may arise from the JOHNSTON-OGSTON effect[3]. Notwithstanding our lack of knowledge concerning the separation of the boundaries, it is evident that the original native DNA has separated into at least two types of molecules, thus showing that different molecules of the total DNA denature under different conditions.

It is thought that the increased charge on denatured DNA[2] (measured at zero ionic strength) is responsible for the differences in sedimentation coefficients observed in this investigation. The highly charged denatured unit will, because of the primary salt effect[4-6], suffer a greater decrease in sedimentation velocity than the native species. Such an effect will not be significant at high ionic strengths.

That low ionic strength does serve to discriminate between native and denatured DNA confirms the recent finding of OTH[7] who reports two boundaries at low ionic strengths. In this case, however, the denaturation originates from low pH, rather than low DNA concentration. SINSHEIMER[8] has found that phage øX174 DNA also exhibits double boundaries at low ionic strength and DNA concentration. Whether this separation indicates the presence of native and denatured molecules is at present uncertain in view of the single-strand structure of this DNA.

The authors wish to acknowledge the financial assistance given to one of us (R.B.I.) by the Anti-Cancer Committee of South Australia, the Commonwealth Scientific and Industrial Research Organisation and Imperial Chemical Industries of Australia and New Zealand Limited.

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Received November 13th, 1959

Biochim. Biophys. Acta, 37 (1960) 162–164

S-adenosylmethionine requirement for plant transmethylations*

It is established that in vertebrates and microorganisms S-adenosylmethionine is the actual methyl donor in enzymic transmethylation reactions which utilize the methyl group of methionine[1]. Transmethylation reactions appear to be prevalent in higher plants as judged by the abundance of N- and O-methyl groups but, while many recent studies have indicated that the methyl moiety of methionine is the source of such methyls, as yet the detailed mechanism of transmethylation in these species has not been clarified. It seemed of interest, therefore, to study a few model reactions of this type. The formation of N-methyltyramine and of hordenine (β-*p*-hydroxyphenethyldimethylamine) appeared suitable since studies *in vivo* indicate that the methyl of hordenine is derived from methionine[2–4].

Extracts of barley seedlings were incubated in the presence of tyramine (or *N*-methyltyramine) as a methyl acceptor and of L-methionine, L-methionine sulfoxide, S-methyl-L-methionine or AMe, each labelled with ^{14}C in the methyl group, as potential donors. Formation of radioactive material extractable into ether from alkaline solution was detected only when the reaction mixture was supplemented with AMe (Table I, Expt. A). Similar results were obtained with extracts of millet seedlings (Table I, Expt. B).

TABLE I

UTILIZATION OF (—)-S-ADENOSYL-L-METHIONINE AS A METHYL DONOR

Additions	*^{14}C in ether phase (counts/min)*			
	Expt. A		*Expt. B*	
	minus enzyme	*plus enzyme*	*minus enzyme*	*plus enzyme*
L-Methionine	6	13	12	10
L-Methionine sulfoxide	0	9	4	4
S-Methyl-L-methionine	13	4	9	4
AMe	24	2,720	60	12,800

Expt. A: Tris(hydroxymethyl)aminomethane, pH 7.6, 100 μmoles; tyramine, 2 μmoles; and barley-extract supernatant were added to each vessel. Either L-[*Me*-^{14}C]methionine, 0.11 μmole, 103·10^3 counts/min; L-[*Me*-^{14}C]methionine sulfoxide (synthesized from L-[*Me*-^{14}C]methionine by treatment with H_2O_2), 0.2 μmole, 127·10^3 counts/min; L-[*Me*-^{14}C]S-methyl-methionine (synthesized from L-[*Me*-^{14}C]methionine[5]), 0.18 μmole, 123·10^3; or [*Me*-^{14}C]AMe (synthesized with partially purified ATP, 3 μmoles, and $MgCl_2$, 3 μmoles. The reaction mixture, final vol. 1.23 ml, was incubated at 25° for 5 h. Trichloroacetic acid was added (followed by enzyme to the control vessels). The supernatant fluid was extracted with ether, made alkaline, and re-extracted with ether. Aliquots of the washed ether extracts of the alkaline solutions were counted. *Expt. B*: Conditions similar to Experiment A with the following changes: N-methyltyramine[7], 1.6 μmoles were present in all vessels. Extracts of millet shoots replaced the barley extracts. ATP and $MgCl_2$ were omitted.

Abbreviations: AMe, (—)-S-adenosyl-L-methionine; ATP, adenosine triphosphate.

* 19th paper in a series on enzymic mechanisms in transmethylation.

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Separate examination of the barley roots and shoots showed that root extracts under these conditions form a single ether-soluble radioactive compound, identified by paper chromatography[8] as N-methyltyramine. The identification was confirmed by adding carrier N-methyltyramine to the radioactive product and recrystallizing repeatedly without significant decrease in specific activity (Table II, Expt. 1). Shoot extracts form an ether-extractable compound identified by chromatography and recrystallization (Table II, Expt. 2) as gramine [3-(dimethylaminomethyl)-indole].

TABLE II

IDENTIFICATION OF THE PRODUCTS OF THE ENZYMIC REACTION

			counts/min/µmole			m.p.	
				Crystals			
Expt.	*Enzyme source*	*Carrier*	*Before crystallization*	*first*	*last*	*Last crystals*	*Literature*
1	Barley roots	N-methyl tyramine	13.1	15.3	17.5 (5)*	118°	130–1°
					19.0 (1)**	233–4°	233–4°
2	Barley shoots	Gramine	—	2.61	3.15 (3)	132–3°	134°
3	Millet shoots	Hordenine***	—	0.67	0.74 (3)	117–8°	117–8°

* Number of crystallizations in parentheses.
** After conversion to β-*p*-hydroxyphenylethyltrimethylammonium iodide.
*** Product was purified by paper chromatography before adding hordenine.

Millet-seedling roots and shoots each formed two compounds, identified paper chromatographically as N-methyltyramine and hordenine[8]. Identification of the hordenine was confirmed as above (Table II, Expt. 3).

The failure of methionine to act as a methyl donor even in the presence of ATP may be ascribed to the specific conditions used since it has been shown that extracts of barley seedlings under suitable conditions will convert methionine to AMe[9].

These experiments show that in three different enzymic transmethylation reactions involved in alkaloid biosynthesis in higher plants AMe functions as a source of the methyl group while other possible methyl donors are completely inactive. It seems warranted, therefore, to suggest that AMe is an important methyl donor in higher plants just as it is in microorganisms and vertebrates.

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Received October 26th, 1959

A requirement for sodium glycocholate in the intestinal conversion of β-carotene to vitamin A *in vivo* and *in vitro*

Although the efficient conversion of *all trans* β-carotene to vitamin A in the intestine of intact mammals has long been recognized[1], the detailed mechanism of this conversion is unknown. Two major problems in studying this transformation have been the difficulty of demonstrating it *in vitro*[2], and the unavailability of adequate methods for accurately measuring minute quantities of vitamin A in the presence of β-carotene, its oxidation products, and other tissue contaminants[2]. Several cases in which the conversion *in vitro* was reported[3,4] could not be confirmed in this laboratory and elsewhere[2,5].

Our investigation of this conversion has been facilitated by the use of uniformly labeled $[^{14}C]$β-carotene isolated from $^{14}CO_2$-grown *Chlorella*[6] and by the unambiguous chromatographic characterization on alumina of the radioactive vitamin A formed —first as the ester and then as the alcohol, aldehyde, and semicarbazone. Artifacts are eliminated by this procedure, and the specific activities of the final vitamin A derivatives remained constant.

Previously it was shown[7] that within 1 h β-carotene in Tween 20 (polyoxyethylene sorbitan monolaurate) solution is converted in 5–15 % yield to vitamin A ester, together with traces of the alcohol and aldehyde, in washed ligated intestinal loops of the rat *in situ*. Further experiments *in vivo* indicated that ligation of the bile duct prevented vitamin A formation in washed duodenal loops, but that the addition of either rat bile or sodium glycocholate to β-carotene suspensions allowed the conversion to occur. In addition, when longitudinally-cut sections of washed rat intestine were incubated *in vitro* in Krebs-Ringer bicarbonate solution with glycocholate, ester formation also took place (Table I). At equivalent concentrations, sodium cholate was about 30 % as effective as glycocholate, but sodium oleate, sodium deoxycholate, and cephalin were ineffective. On the other hand, the formation *in vitro* of vitamin A ester from Tween solutions of $[^{14}C]$vitamin A alcohol or $[^{14}C]$retinene did not require glycocholate.

TABLE I

INTESTINAL CONVERSION OF β-CAROTENE TO VITAMIN A ESTER

10 µg$[^{14}C]$carotene (1000 counts/min/µg) in the indicated detergent solution were injected into washed ligated intestinal loops of bile-duct ligated ether-anaesthetized rats (Expt. II-38) or were incubated with longitudinally-cut intestinal sections in Krebs-Ringer bicarbonate under 95 % O_2–5 % CO_2 at 30° (Expt. II-52). After 1 h the intestine was washed, homogenized in $CHCl_3$–MeOH (2:1) containing 0.1 mg each of β-carotene, vitamin A palmitate, vitamin A alcohol, and retinene. The extract in hexane was chromatographed on alumina.

Expt.	*Condition*	*Detergent*	*Vitamin A ester formed (µg)*
II-38	*In vivo*	Rat bile	0.68
		5 % Tween 20 + 5 % glycocholate	0.66
		5 % Tween 20 + rat bile	0.57
		5 % Tween 20	0.04
II-52	*In vitro*	1.25 % Tween 20 + 0.38 % glycocholate	0.26
		1.25 % Tween 20 + 0.38 % cholate	0.10
		1.25 % Tween 20	0.04

Although a bile requirement has been shown for the utilization of ingested β-carotene and vitamin A in the bile-fistula rat[8], the action of bile has generally been attributed to its emulsifying properties rather than to a specific role for any of its components[9]. The present results suggest that sodium glycocholate, and perhaps other bile salts, are specifically required for the efficient utilization of β-carotene by the intestine. Further studies on the nature of this requirement as well as on the details of the conversion *in vitro* of β-carotene to vitamin A are in progress.

This work was supported by PHS research grant No. A-1278 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service. The [^{14}C]-vitamin A alcohol was most kindly provided by F. Hoffmann-La Roche and Company, Basle, and the Tween detergents by the Atlas Powder Company. The author is indebted to Mrs. Jean Herron for excellent technical assistance.

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Received October 21st, 1959

Biochim. Biophys. Acta, 37 (1960) 166–167

Chymotrypsin inhibitor from *Ascaris*

In 1910 MENDEL AND BLOOD[1] reported that extracts of the body walls of *Ascaris lumbricoides* inhibited the milk-clotting activity of so-called "trypsin". Although COLLIER[2] reported a partial purification of a trypsin- and pepsin-inhibiting substance from *Ascaris*, it was GREEN[3] who first suggested that the inhibition of trypsin and chymotrypsin may be attributed to two independent inhibitors*.

Ascaris lumbricoides var. suis were collected at a slaughter house (the cooperation of the Plankinton Packing Co. is gratefully acknowledged) in the salt medium of BALDWIN AND MOYLE[4]. They were transported to the laboratory, washed and the body walls were dissected away. All subsequent manipulations were carried out at 0° unless otherwise indicated. 100-g portions of body walls (which may be kept frozen for periods of up to one year without loss in activity) were homogenized with 4 vol. cold water in a Waring blendor and extracted for an additional 15 min. The suspension was centrifuged at 25,000 × g for 20 min and then at 105,000 × g for 2 h. The clear red supernatant (Step I) was adjusted to pH 1.9 with 5 N H_2SO_4 and incubated at 37° for 75 min. After cooling to 5°, 5 N NaOH was added to pH 5.7. A heavy precipitate was removed by filtration with the aid of Celite. The inhibitor was precipitated between 0.5 and 0.8 satd. ammonium sulfate[5], at pH 4.7. The precipitate (Step II) was dissolved in water, 0.1 of the volume of Step I and adjusted to a concentration of 10 mg/ml (buiret[6], read through a 1-cm light path, × 31.5 approximates the number of mg protein in the 10 ml of buiret solution). The concentration of trichloroacetic acid was brought to 7.5 % by the addition of 90 % (w/v) trichloroacetic acid. After 5 min the suspension was centrifuged at 25,000 × *g* for 5 min. The clear pale yellow supernatant was brought to a concentration of 20 % trichloroacetic acid by addition of the 90 % (w/v) solution. The precipitate was obtained as described above and immediately suspended in 0.2 *M* phosphate buffer, pH 7.2 (0.2 of the volume of Step II). The suspension was dialyzed against 0.07 *M* acetate, pH 5.15 (Step III). The clarified material was subjected to continuous paper electrophoresis as shown

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* We are grateful to Dr. N. M. GREEN for allowing us to see his manuscript prior to publication.

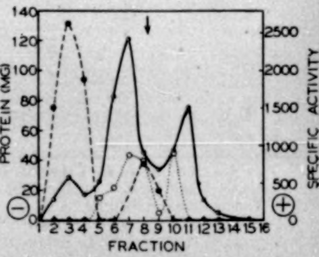

Fig. 1. 520 mg protein accumulated at Step III was introduced on the middle tab of a Beckman/Spinco model CP continuous-flow electrophoresis cell equilibrated against 0.07 *M* acetate buffer, pH 5.15; protein concentration, about 15 mg/ml; charging rate, 1.5 ml/h; applied potential, 500 V. Activity was determined by a method similar to that described by GREEN AND WORK[7] using urea-denatured hemoglobin, either 18 µg chymotrypsin or 6 µg trypsin and a digestion time of 5 min. Specific activity was expressed as µg chymotrypsin or trypsin inhibited/mg protein. ◑—◑, distribution of protein in various fractions; ●—●, specific activity against chymotrypsin; ○—○, specific activity against trypsin; ↓, point at which protein solution was introduced. ⊕ ⊖, orientation of the electrical field.

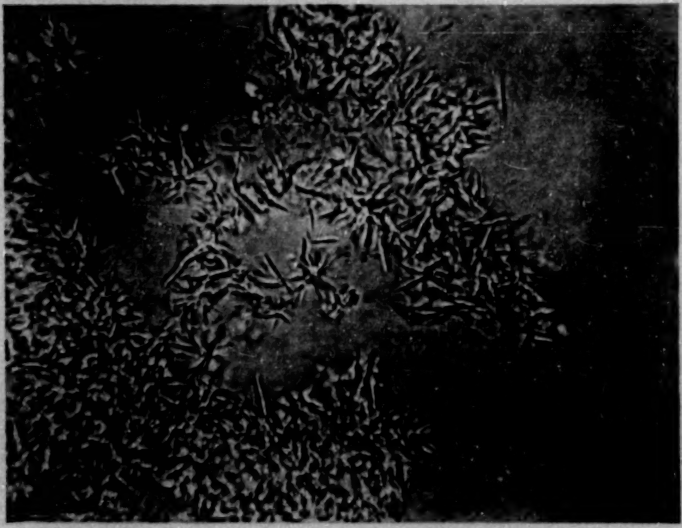

Fig. 2. Crystallized from ammonium sulfate, pH 7.2. Magnification 800 ×.

in Fig. 1. About 20 % of the inhibitory activity against α-chymotrypsin of Step I occurs in Fraction 3 (Step IV). Fraction 3 was lyophilized and then dialyzed against water to reduce the acetate concentration. Visking 18/32 dialysis tubing was selected to minimize loss of the inhibitor. The protein was concentrated to a 10 % solution by lyophilization. An equal volume of a saturated solution of ammonium sulfate was added. The pH was adjusted to 7.2 with 5 *M* ammonia and a drop of 1 *M* phosphate, pH 7.2 was added. A saturated solution of ammonium sulfate was added dropwise to the first signs of silkiness. On standing at room temperature for 8 h, the material crystallized in the form of fine needles as shown in Fig. 2. This preparation represents an increase in specific activity towards α-chymotrypsin of 100-fold over Step I. It inhibits α-chymotrypsin and chymotrypsin B but does not inhibit trypsin.

This work was supported by grants of The National Science Foundation (NSF-G 2430) and the National Institute of Arthritis and Metabolic Diseases (A-535), National Institutes of Health, U.S. Public Health Service. The expert technical assistance of Miss A. KOSLOSKE is gratefully acknowledged.

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Received October 6th, 1959

Biochim. Biophys. Acta, 37 (1960) 167–169

Peptides isolated from peptic hydrolysate of chymotrypsinogen

The continuing interest in our laboratory has been the elucidation of regularities in structure of certain groups of proteins[1], especially the establishment of relations between the two proteases, chymotrypsin and trypsin. Within the scope of this study a number of for the most part lower fragments[2] were isolated from partial acid or tryptic hydrolysates of these proteins or their zymogens.

In order to obtain additional information about the microstructure of chymotrypsinogen the liberation of peptides by peptic degradation of its molecule was studied. Pepsin is a less specific hydrolytic agent than trypsin, and a great number of fragments may therefore be expected to arise from its action on such a large molecule as that of chymotrypsinogen. On the other hand, the advantage of peptic cleavage lies in the fact that the possibility of an autocatalytic destruction of the substrate is excluded. Another aim of the present work was the isolation of peptides containing the residues of tryptophan, which could not be characterized when a partial acid hydrolysate or oxidized chymotrypsin was used as initial materials.

The main experiment involved the cleavage of 6 g chymotrypsinogen, recrystallized five times with ammonium sulphate and twice with ethanol, by crystalline pepsin at 37° and pH 1.9 for 2 h. The enzyme substrate ratio was 1:50. The total uptake of 1 *N* HCl was 3.7 ml. A portion of the hydrolysate corresponding to 5 g of the original protein was brought into a cellophane tubing and dialyzed with agitation using a vibrator. The weights of the non-dialyzing material and of the dialysate after lyophilization were found to be 0.141 g and 4.647 g, respectively. The dialysates were subjected to chromatographic fractionation on a Dowex 50-X2 column of 4 × 152 cm at 40°. An elution gradient of pyridine–formate and pyridine–acetate buffers was used. The mixtures of buffers used tend to favor an effective elution of aromatic peptides from the resin. They can easily be removed from the fractions by lyophilization and they do not impede a direct analysis of ninhydrin-positive substances by paper chromatography. The chromatographic resolution of the peptic hydrolysate was initiated by washing the Dowex 50 column with 20 l 0.2 *M* pyridine brought to pH 3.1 by the addition of formic acid. After this volume had flown through the column an elution gradient of increasing molarity and pH-value was applied. The gradient was developed by the gradual introduction of 25 l 1 *M* pyridine–acetate buffer, pH 5.1, into a mixing vessel (volume 10 l) containing the preceding buffer of pH 3.1. The elution gradient was then continued with 11.6 l 2 *M* pyridine–acetate buffer, pH 5.2. In the next step 5 l 2 *M* pyridine–acetate buffer followed by 2.2 l 2 *M* pyridine were applied to the column. Chromatography was completed by displacing the remaining components with 7.6 l 2 *M* NH_4OH at 25°. Altogether 74.5 l of effluent were collected in 100-ml fractions at an elution rate of 1 fraction–30 min by means of a time fraction collector (rotating intervals of about 25 min) combined with a syphon collecting device. The course of the separation was checked by descending paper chromatography of dry residues of 2-ml aliquots taken from each fraction, in the system *n*-butanol–acetic acid–pyridine–water (15:3:10:12). This procedure used to check the course of separation is much simpler than the ninhydrin method; at the same time the individuality of eluted peptide zones and the possibility of further fractionation by paper chromatography may be judged. After chromatograms had been submitted to visual inspection, corresponding fractions were pooled, and, for the most part, subjected to additional separation by preparative paper chromatography on Whatman No. 1 or 3 paper in the system mentioned. The fractions obtained in this way were further purified by horizontal high-voltage electrophoresis. In a number of fractions which gave no color reaction with the ninhydrin reagent, amino acids definitely corresponding to the original ninhydrin-negative peptides were detected by chromatography after the fractions had been hydrolyzed in 6 *N* HCl. These fractions have not been characterized further at the present time.

Pure peptides obtained by the procedures described were subjected to qualitative analysis and to end-group determinations, used as another criterion of individuality. Peptides which were characterized as above are summarized in Table I.

As obvious from the Table, peptides involving arginine and lysine residues in the middle portion of the chain were isolated from peptic digest of chymotrypsinogen. All of the four residues of arginine present in the molecule of chymotrypsinogen were found in the peptides isolated; of the 13 lysine residues only six have been characterized in the present study. The peptides mentioned are of special importance since they enable longer structures to be deduced by linking together peptides obtained by specific tryptic hydrolysis. The present survey of peptides does not include peptides containing histidine which might be found together with the remaining lysine peptides

TABLE I

PEPTIDES ISOLATED FROM A PEPTIC HYDROLYSATE OF CHYMOTRYPSIN

Glu·(Gly, Ala, Val, Try, Pro, Ser, Glu)
Glu·(Gly, Ala, Val, Try, Pro)·Ser
Glu·(Gly, Ala, Val, Try)

Lys·(Ala, Leu, Thr, Ser, Ileu, Try)·Phe
Lys·(Ala, Leu, Thr)·Ser

Thr·(Arg, Tyr, Asp, Ala, Pro, Leu)
Thr·(Arg, Tyr, Asp)·Ala

Thr·Arg·Tyr

Ala·(Arg, Val, Thr, Ala)·Leu
Ala·(Arg, Val, Thr)·Ala
Ala·Arg·Val

Glu·(Asp, Lys, Gly, Thr)·Phe
Asp·(Lys, Thr, Gly)·Phe

GluNH$_2$·(Glu, Ala, Ser)·Leu
Glu·(Ala, Ser)·Leu
GluNH$_2$·Ala·Ser

Asp·(Ser, Glu)·Ser
Asp·(Ser, Glu)

Ileu·(Asp, Glu, Ser)
Ileu·(Asp, Glu)

Ala·Ser·Leu
Ala·Ser

Ileu·Thr·Leu
Thr·Leu

Asp·(Ser, Gly, Ileu, Lys, Glu, Leu, Ala)·Val
Thr·(Ala, Asp, Thr, Pro, AspNH$_2$, Arg)·Leu
Ala·(Val, Try, Pro, Ser, Glu)·Ser
Asp·(Glu, Thr, Ala, Ileu, Leu, Val)
Leu·(Ala, AspNH$_2$, Lys, Thr, Try)·Ser
Thr·(Pro, AspNH$_2$, Arg, Leu, Glu$_x$, Ala)
Ala·(Ala, Asp, Glu)·Ser
Asp·([I]Leu, Glu, Lys, Ser)·[I]Leu
Ser·(Arg, Ileu, Val, Gly, Asp)
Ser·(Ser, Glu, Thr, Ala, Val)
Val·(Glu, Ser, Thr)·Leu
Lys·(Ileu, Ala)·Val
Ser·(Thr, Ala$_x$)·Ser
Tyr·(Ileu, Val)·Ser
Ala·Ser·Phe
Gly·Val·Ser
Ileu·Val·Ser
Ser·Gly·Leu
Ser·Thr·Ala
Val·Ser·Leu
Ala·(Glu, Asp)
Ala·(Glu, Gly)
Thr·(Asp, Ileu)
(Try, Thr)·Leu
Val·(AspNH$_2$, Try)
Ala·AspNH$_2$
Glu·Ser
Leu·Val
Val·Gly
Val·Thr
Phe·Leu

in that part of the material which was not worked up in the present study. On the other hand, new information concerning tryptophan residues was obtained, since the vicinity of 5 residues of a total of 7 residues present in the molecule was established.

The above work will be published in full in the Collection of Czechoslovak Chemical Communications.

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Received August 26th, 1959

The terminal amino acids of the barium-α_2-glycoproteins of normal human plasma

The low-molecular-weight acidic proteins, prepared from pooled normal human plasma by COHN's method 6[1] or 10[2] and concentrated in Fraction VI[3], include as major components α_1-acid glycoprotein[3,4] and a group of α_2-glycoproteins[5]. On ultracentrifugation the α_2-glycoproteins exhibited similar sedimentation coefficients of approximately 3 S[6]. However, on electrophoresis they revealed two and three components at pH 5.0 and 4.0 respectively[6]. Fractionation of these components was achieved at neutrality by taking advantage of the relative insolubility of the Ba^{++} complex of two of the three α_2-proteins*. Subsequent analysis of the Ba^{++}-precipitable glycoproteins by free electrophoresis demonstrated one component at pH 8.6 and 5.0, but a two-component system at pH 4.2[7]. Ultracentrifugal analysis of the same preparation indicated a single symmetrical peak with comparable sedimentation rates at each of the three mentioned pH values[7].

In this report we wish to present the determination of the terminal amino acids of the Ba^{++}-precipitable α_2-glycoproteins described above and referred to as Ba-α_2-glycoproteins.

The *N-terminal amino acids* were determined according to SANGER's procedure[9]. *Alanine* and *threonine*, as DNP-derivatives, were the only N-terminal amino acids found. A relatively large amount of ε-DNP-lysine and traces of four additional amino acids were noted. Essentially the same results were obtained if the ethanol–ether-denatured, neuraminidase-treated or acetic acid-hydrolyzed[10] form of the Ba-α_2-glycoproteins were analyzed.

The *C-terminal amino acids* were measured by two methods, using carboxypeptidase[9] and hydrazinolysis[11–13]. The native, neuraminidase-treated and acetic acid-hydrolyzed Ba-α_2-glycoproteins, respectively, were digested with this enzyme. Aliquots of the digests were analyzed after different intervals of time of incubation. Leucine was liberated relatively fast followed by threonine and phenylalanine. Again, traces of other amino acids were observed. These results suggested that leucine was most probably terminally located. However, on the basis of this evidence it could not be decided whether threonine and/or phenylalanine were C-terminal amino acids. The second method, hydrazinolysis with hydrazin sulfate as catalyst[13], yielded *leucine* and *threonine* as the only C-terminal amino acids indicating that phenylalanine is not derived from a terminus of a polypeptide chain. These findings were further substantiated by the fact that DNP-phenylalanyl-leucine could be isolated from a dinitrophenylated peptic digest of Ba-α_2-glycoproteins.

The above-described results on the determination of the terminal amino acids together with the observation that the Ba-α_2-glycoprotein fraction was electrophoretically heterogeneous at acid pH values opened the question as to whether this protein fraction contains one protein with two polypeptide chains that would undergo isomerization[14,15] in this pH region, or in fact consists of two very similar proteins with one polypeptide chain each. This problem was resolved by investigating the fraction partially enriched in the faster-moving component of the Ba-α_2-glycoproteins

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Abbreviation: DNP-, dinitrophenyl-.

* The third α_2-glycoprotein was recovered from the supernatant solution, purified and partially characterized[8].

and obtained by free moving-boundary electrophoresis at pH 4.0 in acetate buffer, $\Gamma/2$ 0.1. N-terminal amino acid analysis showed an approximate 3-fold increase in terms of threonine. Thus, this information strongly suggested that the Ba-α_2-glycoprotein fraction consists of two individual proteins with similar composition.

Further details on this study will be published later.

This is publication No. 262 of the Robert W. Lovett Memorial for the Study of Crippling Disease, Department of Medicine, Harvard Medical School, and Massachusetts General Hospital, Boston, Mass. Grants in support of these investigations have been received from the National Institute of Arthritis and Metabolic Diseases (A-2288), National Institutes of Health, U.S. Public Health Service. This work was done under the tenure by K. S. of an Established Investigatorship of the Helen Hay Whitney Foundation, New York City, N.Y.

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Received October 19th, 1959

Biosynthesis of choline *in vitro*

We have recently reported on the isolation of mono- and dimethylaminoethanol from rat-liver phospholipids after the intraperitoneal injection of [*Me*-^{14}C]methionine[1].

In subsequent work it was observed that the incubation of whole rat-liver homogenate with [*Me*-^{14}C]methionine led to the formation of radioactive phospholipids. After hydrolysis, radioactive choline, mono- and dimethylaminoethanol were isolated by column chromatography. It was further found that the formation both of choline and of mono- and dimethylaminoethanol was unaffected by the addition of aminopterin or amethopterin to the incubation mixture, whereas addition of non-radioactive adenosylmethionine completely eliminated the formation of all the radioactive methylated products. The logical conclusion is that all the methyl groups of choline may be derived from adenosylmethionine by transmethylation. Evidence supporting this conclusion is the subject of this communication.

Highly radioactive [*Me*-^{14}C]adenosylmethionine was prepared enzymically according to CANTONI[2]. The purity of the isolated adenosylmethionine was established by paper chromatography with ethanol–water–acetic acid (65 : 34 : 1)[3]. Under ultraviolet light only one spot, which contained all the radioactivity, was found on the chromatogram. No spot corresponding to methionine was present.

The [*Me*-^{14}C]adenosylmethionine was incubated with subfractions of rat-liver homogenate and the incorporation of radioactivity into the phospholipids determined (Table I).

TABLE I

CHOLINE BIOSYNTHESIS IN LIVER SUBFRACTIONS

Liver fraction	*Total counts/min/vessel in phospholipids*
Microsomes (no cephalin)	10,000*
Microsomes, zero time	760
Microsomes	14,100
Mitochondria	6,100
Particle-free supernatant	0
Microsomes + mitochondria + particle-free supernatant	24,100

* Approx. value, corrected for partial loss.

Rat liver was homogenized in 5 parts of 10% sucrose in a glass homogenizer with a Teflon pestle for 1–1.5 min, and centrifuged at 700–800 × *g* for 5 min. Mitochondria were sedimented from the supernatant at about 25,000 × *g* for 10 min, and microsomes at about 100,000 × *g* for 45 min. Mitochondria were washed once. The particulate fractions were suspended in about ¼ of the original volume of 10% sucrose and added to incubations, as was supernatant, in amounts approximating 1 ml of the homogenate freed from cell debris.

Incubation mixture: [*Me*-^{14}C]adenosylmethionine, 0.8 µmole (220,000 counts/min); reduced glutathione, 15 µmoles; tris(hydroxymethyl)aminomethane–HCl buffer, pH 7.25, 100 µmoles; KCl, 30 µmoles; synthetic α-DL-dipalmitoylcephalin (Mann Research Laboratories, New York) 2 mg, (added in a solution of 30 mg crystalline bovine albumin) in total volume of 2.4–2.5 ml. Incubations performed in air for 2 h at 37°.

Reaction was stopped by the addition of 5 ml alcohol–ether (3 : 1) and the mixture heated to boiling. The residues were extracted once more with alcohol–ether and once with ether. The combined extracts were evaporated almost to dryness and the residue extracted with 6–8 ml petroleum ether. The petroleum ether extracts were washed twice with 8 ml 2 *N* NaCl and subsequently concentrated to 0.3–0.5 ml. The phospholipids were precipitated by addition of about 10 vol. of acetone and two drops of 5% $MgCl_2$ in ethanol.

The results show that microsomes alone, incubated with adenosylmethionine, led to the formation of radioactive phospholipids, while no requirement was apparent for either particle-free supernatant fluid or mitochondria. No significant effect was obtained from added synthetic α-DL-dipalmitoylcephalin.

When the isolated phospholipids from a corresponding experiment were hydrolyzed and chromatographed on a column of Dowex 50, only insignificant traces of mono- and dimethylaminoethanol could be detected; 80–90% of the phospholipid radioactivity was recovered in the choline peak. As we had already shown that normal rat-liver phospholipids contain small amounts of dimethylaminoethanol[1], and as it has been maintained that only the third methyl group of choline is incorporated by transmethylation[4], the experiment shown in Fig. 1 was undertaken. A large amount of washed rat-liver microsomes was incubated with a small amount of [*Me*-^{14}C]-adenosylmethionine. Significant amounts of radioactive mono- and dimethylaminoethanol were found, although choline proved to be the main reaction product. These three compounds together accounted for 96.7 % of the radioactivity of the isolated phospholipids. In another experiment the recovery was 88 %. No other radioactive compounds were found.

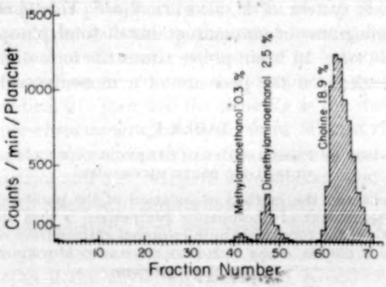

Fig. 1. Methylation of phospholipid aminoethanol from [*Me*-^{14}C]adenosylmethionine in washed rat liver microsomes. One rat liver (9.5 g) was homogenized in 8 parts of 10 % sucrose and centrifuged at 25,000 × *g* for 15 min. The microsome fraction was sedimented from the supernatant fluid by centrifugation at about 100,000 × *g* for 45 min. The particles were washed in 1/3 of the original homogenate volume of 10 % sucrose and finally suspended in 5 ml 10 % sucrose. Incubation mixture: [*Me*-^{14}C]adenosylmethionine, 0.8 µmole (220,000 counts/min); reduced glutathione, 17 µmoles; tris(hydroxymethyl)aminomethane–HCl buffer, pH 7.25, 250 µmoles; KCl, 75 µmoles; microsome suspension, 4.5 ml; total vol. 5.6 ml; incubation time, 2 h at 37°. The phospholipids were isolated as explained in legend for Table I. The phospholipids were hydrolyzed by refluxing in 5 ml 1.2 *N* HCl for 10 h after addition of 3 mg each of nonradioactive choline, mono- and dimethylaminoethanol. The phospholipid bases were separated on a column of Dowex 50 as before[1]. The figure shows the distribution of activity between choline, mono- and dimethylaminoethanol and the % of the added adenosylmethionine methyl recovered in each peak.

On the basis of these results the following conclusions may be drawn: (1) All the methyl groups of choline may be derived from adenosylmethionine by stepwise transmethylation. The introduction of the first methyl group evidently is the rate-limiting step. (2) The microsome fraction of rat liver contains both the necessary enzyme system and a water-insoluble aminoethanol derivative, presumably a phospholipid, that acts as methyl group acceptor in the biosynthesis of choline.

Further studies on this subject are in progress.

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Received October 28th, 1959

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Synthesis of a new phosphatide from monoglyceride and adenosine triphosphate

It was recently shown that phosphatidic acid is synthesized from diglycerides and adenosine triphosphate in dialyzed deoxycholate extracts of brain microsomes[1]. Using the same enzyme preparation but substituting monoglyceride for diglyceride a new radioactive phospholipide spot appeared below the phosphatidic acid in the paper chromatographic system of MARINETTI *et al.*[2]. This spot had not been seen previously on autoradiograms of chromatograms of total phosphatides labeled with ^{32}P either *in vivo* or *in vitro*. In brain preparations the formation of this phosphatide was completely dependent on the presence of a monoglyceride (Table I, Expt. 1).

TABLE I

FORMATION OF PHOSPHATIDES IN DEOXYCHOLATE EXTRACTS OF GUINEA-PIG BRAIN MICROSOMES

The incubation conditions and the method of isolation of the phosphatides were the same as reported previously[1]. The amount of phosphatide synthesized is that obtained from an aliquot representing one-thirtieth of the total yield of microsomal extract from the cerebral hemispheres of one guinea pig. 0.002 *M* diolein, 0.002 *M* monoölein, 0.0002 *M* sodium oleate, 0.0001 *M* CoA, 0.0005 *M* glutathione.

	Quantity of phosphatide synthesized	
	*LPA** *mμmoles*	*PA*** *mμmoles*
Expt. 1		
Control	n.d.***	0.065
Diolein	n.d.	0.280
Monoölein	0.380	0.324
Monoölein + sodium oleate	0.410	0.201
Monoölein + CoA + glutathione	0.610	0.193
Monoölein + sodium oleate + CoA + glutathione	0.650	0.254
Sodium oleate	n.d.	0.067
Sodium oleate + CoA + glutathione	n.d.	0.024
Expt. 2		
Monoölein + sodium oleate	0.114	0.104
Monoölein + sodium oleate + CoA + glutathione	0.230	0.167
Monoölein + sodium oleate + glutathione	0.240	0.183

* Phosphatide presumed to be lysophosphatidic acid.
** Phosphatidic acid.
*** Not detected (the synthesis of 0.03 mμmole would have been detected on the autoradiograms).

Diglyceride or fatty acids plus CoA were ineffective as substrates for the formation of the phosphatide. Higher concentrations of fatty acid than those shown in Table I were inhibitory. CoA plus glutathione enhanced the synthesis of the phosphatide when monoölein was incubated with dialyzed deoxycholate extracts of guinea-pig-brain microsomes. No stimulation was observed with similar preparations from frozen calf brain. The stimulatory effect of CoA plus glutathione in guinea-pig preparations could be obtained with glutathione alone (Table I, Expt. 2), suggesting that the

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Abbreviation: CoA, coenzyme A.

synthesis of the phosphatide was not dependent on CoA. The glutathione effect is presumably due to the protection of sulfhydryl groups on the enzyme. The quantity of the new phosphatide synthesized in the enzyme preparations was insufficient for characterization by conventional means. However, the following experiments indicate that it is a lysophosphatidic acid. Carrier lysophosphatidic acid was synthesized by the method of KABASHIMA[3] and purified by silicic acid column chromatography according to HANAHAN *et al.*[4]. It eluted from the column in the chloroform–methanol (3:2, v/v) fraction. It had a phosphorus-ester ratio of 1:0.98. The unknown radioactive phosphatide could be isolated in the same manner by silicic acid column chromatography. When it was mixed with carrier lysophosphatidic acid and the mixture rechromatographed on the column, the phosphorus peak and the radioactive peak coincided. The radioactive spot had the same R_F as the stained lysophosphatidic acid spot in the paper-chromatographic systems of MARINETTI *et al.*[2], BEISS AND ARMBRUSTER[5] (the spots were elongated in this system), and in butanol–ammonia (equal volumes of butanol and 2 *N* NH_4OH were shaken, and the upper phase was used as the solvent with silicic acid impregnated paper).

The synthesis of phosphatidic acid was always increased on adding monoölein, which suggested that lysophosphatidic acid was a precursor for the phosphatidic acid. This would be expected if the acylating enzyme of KORNBERG AND PRICER[6] were present in the enzyme preparation.

KENNEDY and his associates[7] have presented evidence which indicates that phosphatidic acid is an important intermediate in lipid biosynthesis. The studies of HOKIN AND HOKIN[8,9] indicate that phosphatidic acid plays a role in transmembrane transport. It has already been shown that phosphatidic acid can be formed from α-glycerophosphate and fatty acyl CoA[6] or from diglyceride and adenosine triphosphate[1]. The formation of phosphatidic acid from monoglyceride via lysophosphatidic acid would provide yet another pathway for the synthesis of this important phosphatide. This would also provide a means for direct utilization of monoglyceride for lipid biosynthesis; this would be consonant with the recent studies of SKIPSKY *et al.*[10], who showed that monoglyceride can be absorbed intact from the intestinal tract. It would also be of interest to know whether lysophosphatidic acid might be an intermediate in the synthesis of phosphatides such as lysolecithin.

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Received October 27th, 1959

Extracellular lipid production by a strain of *Rhodotorula graminis*

The properties of the new yeast species *Rhodotorula graminis* were first described by Di Menna[1,2] ,who also reported for the first time the appearance of extracellular lipid in a yeast culture. In the experiment reported below we made use of a strain of *Rhodotorula graminis* (CBS 3043), which was isolated from the leaf surface of citrus plants from Bogor (Indonesia) and Surinam[3]. This red yeast is able to produce large quantities of intra- and extra-cellular lipids on malt agar and in liquid media. The following experiment was carried out in a liquid medium containing 3.5–4.0 % glucose, 0.1 % KH_2PO_4, 0.1 % $(NH_4)_2SO_4$, and 0.02 % $MgSO_4$. Shake cultures in 5-l Erlenmeyer flasks, containing 1 l of this medium, were inoculated with yeast cells and incubated at 29°. The decrease of the pH, due to the uptake of the ammonium nitrogen by the yeast cells, was neutralized by the periodical addition of some sterile KOH solution, the pH thus being kept at about 4.5. The glucose was used up in 5–6 days and the yeast was separated from the culture liquid by centrifugation. The supernatant had a milky appearance, which was due to many minute fat droplets (diameter 0.1–2 μ) in the liquid.

Fig. 1 describes the yeast growth, increase of viable cells, nitrogen uptake and fat formation as a function of glucose consumption. The number of viable cells increased only in the beginning of the experiment. The fat synthesis started when the nitrogen had been used up and proceeded practically linearly with the glucose consumption. The quantity of yeast fat can amount to 3–4 g/l culture medium, and 2 g/l medium can be extracted from the extracellular fluid with ethyl ether. The lipids of the yeast cells and those of the culture medium were analysed by means of gas–liquid chromatography[4,5]. The intracellular fat had a normal composition, compared with that of other yeasts[6] (see Table I). The composition of the lipids present in the culture medium (pH 4.5), however, differed considerably from normal yeast fat, and their analysis proved to be difficult. The lipids were hydrolysed with alkali and after acidification about 60 % of the original weight of the lipids was obtained as higher

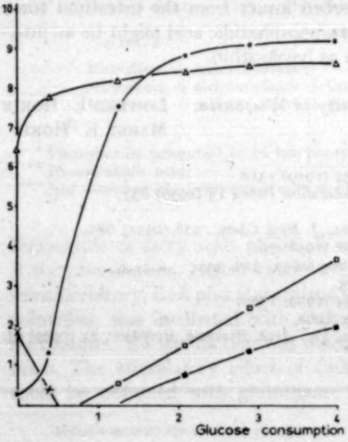

Fig. 1. The abscissa shows the sugar consumption (g/100 ml culture liquid). ×—×, amount of N (mg/10 ml) in the culture liquid; ○—○, yeast growth (g/l); △—△, log viable cells in 1 ml culture medium; □—□, yeast fat (g/l); ■—■, extracellular lipids (g/l).

TABLE I

COMPOSITION OF THE INTRACELLULAR FAT OF *Rhodotorula graminis* GROWN IN THE NORMAL GLUCOSE MEDIUM OF pH 4.5

Fatty acids	*% (by weight)*
Myristic acid	0.5
Lauric acid	1.0
Palmitic acid	22.5
Stearic acid	1.0
Palmitoleic acid	3.5
Oleic acid	63.5
Linoleic acid	6.5
Not yet identified*	1.5
Iodine value (exp.)	72.0
Iodine value (calc. from the data of the chromatographic analysis)	71.0

* This acid forms a peak situated just before that of behenic acid.

fatty acids, separating as an oily layer. In the aqueous solution, the rest of the lipids was identified as acetic acid (about 35 %) and poly-alcohols. The higher fatty acids of these lipids were analysed on a column of Celite 545, impregnated with 20 % polyglycolglutarate at a temperature of 210°, with hydrogen as carrier gas. The chromatogram obtained was very complicated and most of the peaks were not completely separated. Three groups could be distinguished: (1), a group around the peak of palmitic acid (corresponding to about 15 % of the total amount of injected fatty acids); (2), a group around the peak of stearic acid (about 60 %) and (3) a group just before behenic acid (15 %). An exact interpretation of this chromatogram has not been possible so far.

With the aid of paper-chromatography[7] it was found that the poly-alcohols in the yeast fat were glycerol and sorbitol; in the lipids from the culture medium sorbitol and arabitol could be detected, but no glycerol was present.

Other experiments in shake cultures were carried out with this strain of *Rhodotorula graminis* to study the influence of pH, temperature and other factors on fat synthesis. The extra-cellular lipid production was highest at a pH of 4.5–6.5; at a pH of 4.0 and higher than 7.0 much lower values were obtained.

At 18° extracellular lipid production was also considerably higher than at 29°, but yeast growth and intracellular fat synthesis were much lower at the lower temperature. The composition of the intra- and extra-cellular lipids showed also differences at various pH values and temperatures. More detailed information on these experiments with *Rhodotorula graminis* and other strains of yeast, which produce extracellular lipids, will be described elsewhere[8].

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Received October 13th, 1959

An enzymic defect in fatty acid synthesis by alloxan-diabetic rat liver*

In mammalian tissues, fatty acids are not synthesized by simple reversal of the fatty acid oxidation cycle outlined by LYNEN[1]. The acyl-CoA dehydrogenase step is apparently not reversible under physiological conditions. The reduction of α, β-unsaturated acyl-CoA derivatives is catalyzed by a TPNH-specific enzyme present in the particle fraction of liver[2]. Since fatty acid oxidation is not impaired in diabetic rat liver, but lipogenesis is severely depressed, one of us*, at the suggestion of Professor F. LYNEN, investigated the activity of this TPNH-specific enzyme in normal and in diabetic rat-liver homogenates.

Homogenates were prepared from livers of alloxan-diabetic rats in 0.25 M sucrose (10 ml/g liver) and fractionated into mitochondria, microsomes, and particle-free supernatant. Mitochondria and microsomes were recentrifuged once in 0.25 M sucrose, and resuspended in 0.1 M phosphate buffer (pH 7.5) with 0.5 % Na-cholate. TPNH was prepared with glucose-6-phosphate dehydrogenase, crotonyl-CoA from crotonic anhydride, and CoA by the DECKER[3] modification of the method of SIMON AND SHEMIN[4]. Protein was determined by the biuret method[5].

The assay system contained 500 µmoles Tris (pH 7.4), 50 µmoles $MgCl_2$, 1 µmole TPNH, and 0.1–0.2 ml homogenate fraction in a volume of 1.6 ml. The reaction was started by addition of 0.2 µmole crotonyl–CoA to one cuvette and an equivalent amount of water to a blank which contained all components except the CoA derivative. The linear decrease in absorption at 366 mµ during the first 5–10 min after addition of the substrate, measured against the blank value in an Eppendorf photometer, was used for calculating activity of reducing enzyme.

More than two-thirds of the activity of the α,β-unsaturated acyl-CoA reducing enzyme was in the microsomal fraction, and about one-fourth in mitochondria. The particle-free supernatant showed almost no TPNH oxidation in the presence of crotonyl-CoA. In the diabetic preparations, the activity of this reducing enzyme in the microsomal fraction was 30 % of normal and in mitochondria, 20 % of normal (Table I).

As most of the activity of this enzyme was found in the microsomal fraction, we studied the effect of microsomes on the fatty acid synthesis by liver supernatants of normal and diabetic rats. The method previously described[6] was followed except

TABLE I

TPNH-DEPENDENT REDUCTION OF CROTONYL-CoA BY PARTICLE FRACTIONS OF NORMAL AND DIABETIC RAT-LIVER HOMOGENATES

Values are mµmoles TPNH oxidized/mg particle protein/min and standard errors.

Condition of animal	*No. of expts.*	*Mitochondria*	*Microsomes*
Normal	10	2.27 ± 0.07	5.15 ± 0.21
Diabetic	13	0.45 ± 0.06	1.61 ± 0.11

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* Part of this work was done in the Institut für Biochemie, Universität München, Germany (K.J.M.).

Abbreviations: CoA, coenzyme A; ATP, adenosine triphosphate, DPN and TPN, di- and triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

TABLE II

STIMULATION OF FATTY ACID SYNTHESIS IN NORMAL AND DIABETIC RAT-LIVER SUPERNATANT FRACTIONS BY THE ADDITION OF MICROSOMES

6.2 µmoles ($5 \cdot 10^5$ counts/min) [1-^{14}C]acetate were incubated with 1.5 ml supernatant and 0.1 ml microsomes in 0.15 *M* glycylglycine buffer (pH 7.5) with 60 µmoles reduced glutathione, 70 µmoles $MgCl_2$, 48 µmoles ATP, 1 µmole DPN, 1 µmole TPN, 0.2 µmole CoA, 75 µmoles citrate, 40 µmoles glucose-6-phosphate, and 10 µmoles $KHCO_3$ in a final volume of 3.6 ml at 30° for 2 h in air. Values are mµmoles acetate converted to fatty acids/mg supernatant protein, and standard errors.

Condition of animal	*No. of expts.*	*Supernatant*	*Supernatant + microsomes*	*Microsomal stimulation*
Normal	5	0.98 ± 0.28	7.60 ± 1.30	7.75
Diabetic	4	0.93 ± 0.16	2.10 ± 0.33	2.25

that 0.25 *M* sucrose was used as homogenizing medium instead of 0.1 *M* phosphate buffer. Fatty acid synthesis from acetate in the normal supernatant fraction was increased 7-fold by the addition of normal microsomes, whereas with the diabetic system, this stimulation was only 2-fold even though the production of fatty acids from acetate was about the same in normal and diabetic supernatant fractions alone (Table II). Although we occasionally observed stimulations, the effect of mitochondrial additions to the supernatant fractions was variable. However, DITURI AND GURIN[7] have shown that aqueous extracts of liver mitochondria do stimulate fatty acid synthesis from acetate in particle-free supernatant fractions.

Since the activity of the fatty acid synthesizing enzyme system and the activity of the α,β-unsaturated acyl-CoA reducing enzyme in these diabetic preparations are 30% of those observed in normal preparations, it seems reasonable to assume that the decreased activity of this enzyme is a principal cause for the defective fatty acid synthesis in the diabetic rat liver.

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Received September 28th, 1959

Biochim. Biophys. Acta, 37 (1960) 180–181

Inhibition of lipoyl dehydrogenase by trace metals

In previous communications[1,2] it has been reported that lipoyl dehydrogenase of pig-heart muscle is a flavoprotein, and furthermore that this enzyme is identical with the classical diaphorase of STRAUB[3] and of SAVAGE[4]. This conclusion has been confirmed by SEARLS AND SANADI[5]. Furthermore, NOTANI AND GUNSALUS[6] have reported that the lipoyl dehydrogenases from *Escherichia coli* and *Leuconostoc mesenteroides* both showed very high "diaphorase" activity, *i.e.* catalysed oxidation of DPNH by artificial electron acceptors. As discussed elsewhere[7,8] the physiological

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Abbreviations: DPN^+, DPNH, oxidised and reduced diphosphopyridine nucleotide; $lipS_2$, oxidised lipoic acid; EDTA, ethylenediaminetetraacetate; DCIP, 2,6-dichlorophenolindophenol,

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role of STRAUB diaphorase would thus appear to be to produce DPNH from DPN+ in the oxidative decarboxylation of keto acids, and not to act as a DPNH-oxidising enzyme in the respiratory chain, as previously claimed[9]. This conclusion has been challenged by GREEN *et al.*[10], who still maintain that diaphorase, in a lipid complex, is the physiological DPNH-oxidising enzyme. As an argument to support this conclusion they claim that diaphorase isolated by the original method of STRAUB does not possess any lipoyl dehydrogenase activity. The present communication reports that, when diaphorase is prepared carefully, it has full lipoyl dehydrogenase activity. However, when precautions are not taken to exclude trace metals, extensive inactivation occurs toward lipS$_2$ as electron acceptor, accompanied by an enormous increase in reactivity toward the artificial acceptor DCIP.

This finding arose from observations that on dialysis pure enzyme frequently became inactivated toward lipS$_2$ and activated with respect to DCIP. This did not occur when the dialysis tubing was washed with dilute EDTA before use (soaked for 15–30 min with 10^{-3} *M* EDTA) suggesting that a trace-metal effect was involved. That small amounts of metal ions can alter the enzyme in this way is illustrated in Fig. 1, which describes the effect of incubating ultracentrifugally pure diaphorase at 0° with 1 atom Cu^{++}/mole enzyme. It is seen that in time there is an almost complete loss of lipoyl dehydrogenase activity, accompanied by a 20-fold increase in the activity toward DCIP. It should be noted that the specific activity of untreated enzyme with lipS$_2$ is higher than originally quoted[1,2]. This is due partly to the inclusion of 10^{-3} *M* EDTA in the assay system, and partly to the presence of a previously unrecognized inhibitor in certain commercial samples of DPNH. The DPNH used in this work was a sample from the Pabst Laboratories. The possible nature of this inhibitor will be considered in a later communication. Secondly, it should be noted that the specific activities for DCIP are very much lower than those previously

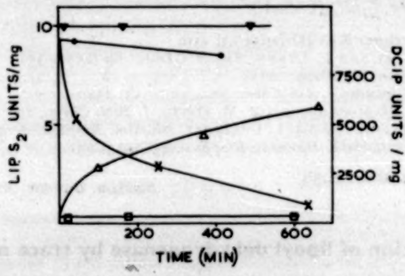

Fig. 1. Pure diaphorase ($6 \cdot 10^{-6}$ *M*) was incubated at 0° with or without $6 \cdot 10^{-6}$ *M* CuSO$_4$, in the presence of 0.02 *M* phosphate, pH 6.3. LipS$_2$ activity was determined spectrophotometrically at 25° by following the change in absorbancy at 340 mμ (1 cm light path) due to oxidation of DPNH. Each cell contained 150 μmoles phosphate buffer (pH 5.9), 2 μmoles DL-lipoic acid, 0.6 μmole DPNH, 3 μmoles EDTA and 2 mg bovine serum albumin in a volume of 3 ml. Protein concentration was measured by absorbancy at 280 mμ ($E^{1\,\text{cm}}_{1\,\text{mg/ml}} = 1$). DCIP activity was determined at 600 mμ under the same conditions, except that pH was 7.2 and the lipS$_2$ was replaced by 0.12 μmole DCIP. It should be noted that whereas the unit of activity with lipS$_2$ represents an absorbancy change of 1.0/min, the DCIP unit (following SAVAGE[4]) represents an absorbancy change of 0.01/min. ●, lipS$_2$ control, no Cu^{++}; ▽, lipS$_2$ control, no Cu^{++}, but with 10^{-3} *M* EDTA; ×, lipS$_2$, 1.0 atom Cu^{++}/mole enzyme; □, DCIP control, no Cu^{++} △, DCIP, 1.0 atom Cu^{++}/mole enzyme.

reported by Savage[4] and by Massey[2]. Again this is due to the inclusion of bovine serum albumin and EDTA in the assay system. It was noted that, when the assay with DCIP was performed by the method of Savage[4], there was a considerable lag period in the reduction of DCIP, with initial rates of about 0.1–0.2 of the maximum obtained. When the reaction was carried out in the presence of serum albumin and EDTA, the initial rate was slightly higher than when these components were absent, but now no activation during the course of the assay was observed. On activation by Cu^{++} as described in Fig. 1, this lag period in the absence of serum albumin and EDTA was no longer observed, and the rates obtained were the same whether or not these components were present. It is thus concluded that the presence of serum albumin and EDTA is necessary in order to measure the true activity of the native enzyme toward DCIP, and that the activities previously reported are too high because of the effect of trace metals prior to or during the assay. It must be emphasised that the effects shown in Fig. 1 are produced catalytically by the added Cu^{++}, since the same results were obtained in a longer time with 0.5 atom Cu^{++}/mole enzyme. The copper effect is apparently due to catalytic oxidation of essential –SH groups, since incubation of the enzyme with arsenite and DPNH produces the same effects, *i.e.* an extensive inhibition of the lipoyl dehydrogenase activity and a great increase in the DCIP activity.

It seems likely that the inability of Ziegler *et al.*[10] to observe lipoyl dehydrogenase activity with Straub diaphorase is due to a trace-metal effect of the type described above.

The lipoyl dehydrogenase activity of a preparation of diaphorase made by the method of Straub was 5.8 units/mg. Further purification by heating to 70° for 5 min, followed by $(NH_4)_2SO_4$ fractionation gave a preparation with a specific activity of 9.6, compared with 10.0 obtained with the pure enzyme obtained with a column of calcium phosphate gel[1,2]. Similarly, the specific activities with $K_3Fe(CN)_6$ were 100 and 110, respectively, and with DCIP 200 and 250, respectively. It is thus clear that the enzymes isolated by the mild method previously described[1,2] and by that of Straub[3] are identical, and function as lipoyl dehydrogenase.

One of us (C.V.) is working under the auspices of The Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the advancement of Pure Research (Z.W.O.).

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Received October 6th, 1959

On a previously unknown reaction of the D-glyceraldehyde-3-phosphate dehydrogenase

It is well known that D-glyceraldehyde 3-phosphate dehydrogenase is able to catalyze many reactions.

Table I shows that reduction of DPN can be observed in a reaction mixture that contains no D-glyceraldehyde 3-phosphate or other oxidizable substrates but contains ATP, IMP, arsenate, the enzyme and coenzyme. The reaction proceeds also in the presence of only bound coenzyme.

TABLE I

REDUCTION OF DPN BY GLYCERALDEHYDE 3-PHOSPHATE IN ABSENCE OF SUBSTRATE

Components of reaction mixtures	*1*	*2*	*3*	*4*	*5*	*6*
PGAD(DPN)$_3$ (0.5 mg/ml)	—	+	+	+	+	+
DPN (0.33 mg/ml)	+	—	+	+	+	+
ATP (1.9 mg/ml)	+	+	—	+	+	+
IMP (approx. 0.02 mg/ml)	+	+	+	—	+	+
Arsenate (0.186 mg/ml)	+	+	+	+	—	+
Increase in extinction at 340 mμ in 10 min	—	0.042	—	—	—	0.108

The reaction has been studied with 4-times recrystallized beef-muscle PGAD isolated according to ELODI AND SZÖRÉNYI[1], but it has been also demonstrated with crystalline enzymes isolated from swine and rabbit muscle[1] and also with PGAD's isolated from yeast[2].

The rate of the reaction increases according to the increase in the concentration of the individual components up to saturation. The reaction proceeds also when instead of arsenate, phosphate or borate are used, but in these cases the rate of the reaction is much smaller. Higher concentrations of these ions inhibit the reaction.

The pH optimum of the enzymic process is 8.1–8.3. The product of the reaction was isolated from the reaction mixture and found to have the same spectrum as DPNH. When acetaldehyde and alcohol dehydrogenase were added to the reaction mixture when the reaction had stopped, the absorbance at 340 mμ decreased to the starting value (Fig. 1).

Applying KALCKAR's enzymic method[3], it was found that the ATP was decomposed during the reaction and ADP was formed. The phosphate acceptor of the reaction is probably IMP, which is presumably phosphorylated to IDP. Neither ADP nor AMP act as a phosphate acceptor in this reaction.

As measured with the spectrophotometric method of BOYER[4], some of the SH groups of the enzyme disappear during the reaction. It has been found that there is a close connection and definite quantitative relation between two apparently independent reactions—namely the phosphate transfer and the oxido-reduction. The phosphate transfer does not take place without oxido-reduction and *vice versa*. The number of DPN molecules reduced equals the number of –SH groups of the enzyme which

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Abbreviations: PGAD D-glyceraldehyde-3-phosphate dehydrogenase; ADH, alcohol dehydrogenase; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; IMP, inosine monophosphate.

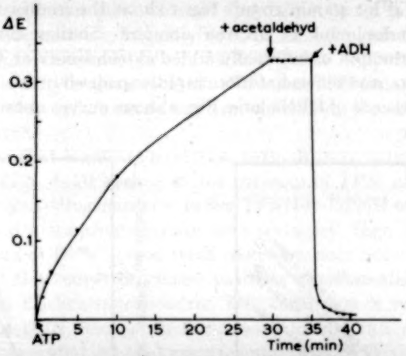

Fig. 1. Reduction of DPN by glyceraldehyde-3-phosphate dehydrogenase and oxidation by acetaldehyde and ADH.

are oxidized, and twice as many ATP molecules give their terminal phosphate to the IMP.

The results will be published in detail in the *Acta Physiologica Hungarica.*

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Received October 6th, 1959

Biochim. Biophys. Acta, 37 (1960) 184–185

Co-factor requirement of isocitric dehydrogenase in Ehrlich-ascites-cell mitochondria

Convincing evidence has been adduced to show that in rat-liver mitochondria and -heart sarcosomes depleted of pyridine nucleotides, isocitrate is oxidised via a TPN-dependent dehydrogenase yielding TPNH which is oxidised via a transhydrogenase and the DPNH-diaphorase[1,2]. Since it is doubtful whether this pathway operates in tumours, we have investigated the co-factor requirement for isocitrate oxidation by Ehrlich-ascites-cell mitochondria.

Mitochondria were isolated by the method of HAWTREY AND SILK[3] and were depleted of pyridine nucleotides by incubation in 0.25 *M* sucrose with 0.03 *M* phos-

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Abbreviations: DPN, DPNH, oxidised and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidised and reduced triphosphopyridine nucleotide; AMP, ADP, adenosine mono- and di-phosphate.

phate buffer (pH 7.4) for 40 min at 30°. Fig. 1 shows the results of experiments using 2,6-dichlorophenolindophenol as electron acceptor. Similar curves were obtained by following the reduction of externally added cytochrome *c* at 550 mμ, except that in this case isocitrate was somewhat more rapidly oxidised in the presence of DPN + TPN than in the presence of DPN alone. Fig. 2 shows curves obtained manometrically with O$_2$ as acceptor.

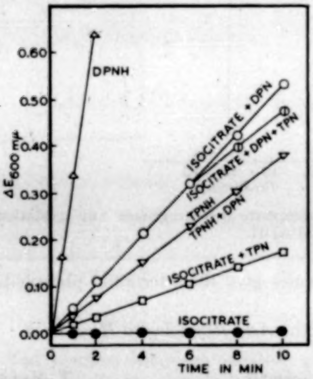

Fig. 1. Reduction of 2,6-dichlorophenolindophenol by pyridine-nucleotide-depleted Ehrlich-ascites-tumour mitochondria. Reaction mixture in a final volume of 3.0 ml: K, Na phosphate buffer (pH 7.4), 0.10 *M*; KCN, 0.001 *M*; MgCl$_2$, 0.003 *M*; DL-isocitrate (K$^+$ salt), 0.0023 *M*; nicotinamide, 0.02 *M*; 2,6-dichlorophenolindophenol, 50 μg; depleted mitochondria (0.26 mg protein); DPN and TPN, 400 μg each; DPNH, generated from DPN (400 μg) by addition of ethanol (0.04 ml) and alcohol dehydrogenase (0.5 mg) to the reaction mixture; TPNH, generated from TPN (400 μg) by addition of glucose-6-phosphate (0.0017 *M*) and glucose-6-phosphate dehydrogenase (1.0 mg) to the reaction mixture.

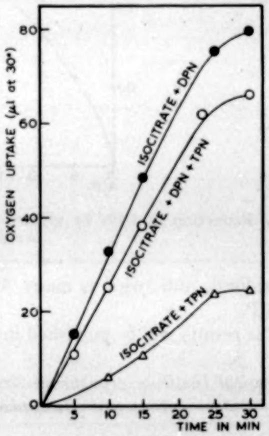

Fig. 2. Oxygen uptake of pyridine-nucleotide-depleted Ehrlich-ascites-tumour mitochondria. Reaction mixture in a final volume of 3.0 ml: K, Na phosphate buffer (pH 7.4), 0.045 M; D-glucose, 0.034 *M*; KF, 0.015 *M*; MgCl$_2$, 0.008 *M*; ADP, 0.0025 *M*; AMP, 0.001 *M*; ethylenediaminetetraacetic acid (pH 7.4), 0.002 *M*; KCl, 0.01 *M*; sucrose, 0.067 *M*; DL-isocitrate, 0.0033 *M*; cytochrome *c*, $9.8 \cdot 10^{-6}$ *M*; hexokinase (crystalline), 1.5 mg; depleted mitochondria (2.02 mg protein); DPN and TPN, $1.2 \cdot 10^{-3}$ *M*. Temp. 30°.

Isocitrate was not oxidised in the absence of added DPN or TPN as expected with mitochondria depleted of pyridine nucleotide (Fig. 1), but oxidation occurred rapidly in the presence of DPN and, at a slower rate, in the presence of TPN with all three acceptors. It would thus seem that isocitric dehydrogenase in Ehrlich mitochondria can function with either DPN or TPN as co-factor.

Possibly the DPN and TPN might compete for the co-factor site on a single dehydrogenase, or alternatively both a DPN- and a TPN-specific isocitric dehydrogenase may be present in the tumour mitochondria. A lower rate of isocitrate oxidation in the presence of DPN + TPN than in the presence of DPN alone (Fig. 2) might indicate competition between the co-factors, whereas a greater rate with DPN + TPN than with DPN alone (as with cytochrome *c*) would suggest the existence of separate DPN and TPN isocitric dehydrogenases. Mitochondria aged 50 h at 2° after pyridine nucleotide depletion show a loss of approximately 63 % in DPN-dependent isocitric dehydrogenase activity, but only 10 % in TPN-dependent activity as measured with 2,6-dichlorophenolindophenol. Such evidence supports the postulate of two separate isocitric dehydrogenases. Both DPN- and TPN-linked isocitric dehydrogenases have been observed in ox-adrenal mitochondria, the DPN-linked enzyme being the more active[4].

The observation that isocitrate oxidation with all three acceptors is more rapid in the presence of TPN + DPN than in the presence of TPN alone would suggest the operation of a transhydrogenase converting TPNH to DPNH which is very rapidly oxidised. However, if a transhydrogenase were necessary, then no oxidation would occur in the presence of DPN[1]. Since rapid oxidation does occur in the presence of DPN, it seems that the transhydrogenase pathway is either absent or of little importance in depleted Ehrlich mitochondria. This conclusion is substantiated by the fact that addition of DPN does not increase the oxidation rate of externally added TPNH (Fig. 1). On somewhat different experimental grounds Williams-Ashman[5] also concluded that transhydrogenase activity is absent in Ehrlich cells, while Reinafarje and Potter[6] failed to detect the enzyme in Novikoff-hepatoma cells. Findings regarding transhydrogenase activity in a variety of cell types have been summarised by Kit and Griffin[7].

The curves with 2,6-dichlorophenolindophenol and cytochrome *c* as acceptor acceptor clearly indicate the presence of a TPNH-oxidation pathway in depleted ascites mitochondria. In both cases externally added TPNH is oxidised at a greater rate than TPNH generated by isocitrate oxidation, indicating that the dehydrogenase reaction is rate limiting in TPN-dependent isocitrate oxidation. Since the oxidation rates of external and isocitrate-generated TPNH are both lower with cytochrome *c* as acceptor than with 2,6-dichlorophenolindophenol, it would seem that a further rate limiting step may occur between cytochrome *c* and the flavin at which 2,6-dichlorophenolindophenol accepts electrons. TPNH-cytochrome *c* reductase activity has been reported in the Novikoff-hepatoma, hyperplastic epidermis, papillomas and carcinomas[8,9].

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Received August 18th, 1959

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Thyroxine-enhanced oxidative phosphorylation of rat-liver mitochondria

During the past ten years many investigators have reported that thyroxine was capable of uncoupling mitochondrial oxidative phosphorylation. Recently **Bronk**[1] reported that thyroxine-treated mitochondrial "digitonin-particles" yielded elevated P/O ratios.

In the present communication we shall report our findings concerning the effect of varying the concentration of L-thyroxine on the P/O ratio of intact rat-liver mitochondria. We have found that 10^{-4} to 10^{-3} M L-thyroxine uncouples oxidative phosphorylation. Also we have found that 10^{-5} M L-thyroxine elevates the P/O ratio about 60% when β-hydroxybutyrate was the substrate and about 100% when reduced cytochrome *c* was the substrate.

Liver mitochondria were isolated from normal male Sprague-Dawley rats by a standard procedure[2]. The system that was used to study the effect of thyroxine on mitochondrial P/O ratios was almost identical to that described by **Tapley and Cooper**[3]. Mitochondria were preincubated in hypotonic sucrose for 10 min as an initial step in all experiments. Oxygen consumption was measured by the Warburg apparatus and phosphate esterification was determined by difference.

In Fig. 1 it can be seen that "very low" concentrations of L-thyroxine have essentially no effect on the P/O ratio resulting from β-hydroxybutyrate oxidation. When the concentration of L-thyroxine was raised above 10^{-6} M the resulting P/O ratios were increased with a maximum at 10^{-5} M. As the concentration was further increased to 10^{-3} M, uncoupling was found to occur. The observed change in P/O ratios was *always* due to a change in the utilization of inorganic phosphate, oxidation remaining unchanged.

When ascorbic acid-reduced cytochrome *c* was used as the substrate in essentially

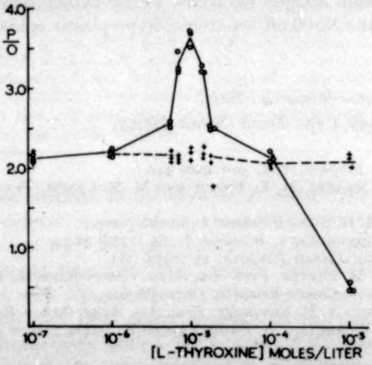

Fig. 1. Effect of L-thyroxine on rat-liver mitochondrial P/O ratios. Each reaction vessel contained, in a total vol. of 3 ml, 0.045 μmole cytochrome *c*, 3.0 μmoles diphosphopyridine nucleotide, 15.1 μmoles adenosine diphosphate. 15.0 μmoles $MgCl_2$, 30.0 μmoles NaF, 37.5 μmoles KH_2PO_4, 40.0 μmoles β-hydroxybutyrate, 1.0 ml mitochondria, suspended in 0.1 M tris(hydroxymethyl) aminomethane buffer, pH 7.4 containing an amount derived from 0.5 g wet weight of liver. (+) = Control and (o) = with L-thyroxine.

the same system it was found that 10^{-5} *M* L-thyroxine enhanced the P/O value from a normal value of 0.55 to 1.10. Again, the increase was due *only* to increased inorganic phosphate utilization, oxidation remaining the same for the control and the thyroxine-treated system.

It is concluded that within a fairly critical concentration range L-thyroxine causes a "tighter coupling" of the phosphorylative reactions associated with rat-liver mitochondrial oxidative phosphorylation. This increased "energetic efficiency" is coupled to the oxidation of ferrocytochrome *c* by oxygen. These results indicate that the maximum amount of mitochondrial adenosine triphosphate that can be formed by passage of a pair of electrons from DPNH to oxygen is not *three* but more likely *four* molecules.

This study was aided by grants from the American Heart Association, the National Science Foundation (G-1157), the U.S. Public Health Service (H-2102C) and the Kentucky Heart Association.

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Received October 17th, 1959

Biochim. Biophys. Acta, 37 (1960) 188–189

Activation of iodine utilization in thyroid-gland homogenates by cytochrome *c* and quinones

In a previous report we described the incorporation of ^{131}I[iodide] into iodoproteins by cell-free preparations of sheep-thyroid tissue and the activation of these preparations by flavin cofactors[1]. The present communication presents the results of further experiments dealing, in particular, with the activation by cytochrome *c* and various quinones.

Cell-free tissue preparations were prepared from sheep-thyroid glands and incubated with ^{131}I$^-$ as described previously[1]. Cytochrome *c* was added to the incubation mixture in dry crystalline form and quinones in 20 µl of ethanol solution. The incubations were terminated by addition of propylthiouracil, and the samples were analyzed by chromatography on filter paper[2].

The results shown in Table I indicate that the incorporation of ^{131}I$^-$ into protein by the particulate (mitochondrial-microsomal) fraction was augmented by incubating with 0.0005 *M* FMN or menadione. Cytochrome *c* had no effect. Interestingly enough, when the particulate material was incubated in the presence of the soluble fraction (100,000 × *g* supernatant), cytochrome *c* definitely activated ^{131}I-utilization; under these conditions FMN was inactive. Thus, the soluble fraction appears to contain a component(s) that is necessary for cytochrome *c* action and that blocks FMN action.

Abbreviation: FMN, flavin mononucleotide.

TABLE I

EFFECTS OF FMN, CYTOCHROME *c* AND MENADIONE ON [^{131}I]PROTEIN FORMATION BY VARIOUS CELL-FREE PREPARATIONS OF SHEEP THYROID TISSUE

(Each sample consisted of 2 ml tissue preparation incubated for 2 h at 37° with 10–20 μC ^{131}I. All supplements present at $5 \cdot 10^{-4}$ *M* concentrations.)

Tissue preparation used	*Supplements added*	*% total ^{131}I incorporated into proteins*
Particulate fraction*	None	10.6
Particulate fraction*	FMN	57.8
Particulate fraction*	Cytochrome *c*	4.3
Particulate fraction*	Menadione	69.0
Soluble fraction	None	0.4
Soluble fraction	FMN	0.9
Soluble fraction	Cytochrome *c*	0.6
Soluble fraction	Menadione	2.1
Particulate + soluble	None	11.1
Particulate + soluble	FMN	9.8
Particulate + soluble	Cytochrome *c*	19.5
Particulate + soluble	Menadione	22.2
Particulate + boiled** soluble fraction	None	4.3
Particulate + boiled** soluble fraction	FMN	46.8
Particulate + boiled** soluble fraction	Cytochrome *c*	3.1
Particulate + boiled** soluble fraction	Menadione	34.3

* Mitochondria and microsomes suspended in Krebs-Ringer bicarbonate buffer containing 200 μg I^-/100 ml.

** 1.5 min in boiling-water bath.

The results of the experiments with particulate matter suspended in heat-treated soluble fraction indicate that this component(s) is destroyed by heat.

The activation of the thyroid particulate fraction by FMN is reported to require light[3]. We have confirmed this finding, but its significance is not clear. FMN does not promote iodination of proteins in whole homogenates of various non-thyroid tissues such as kidney, liver, mammary, gland, spleen, salivary gland and testis, or in control systems composed of solutions of crystalline serum albumin, pepsin or insulin.

The effect of cytochrome *c* was not duplicated by hemoglobin or hemin. As in the case of FMN, cytochrome *c* did not promote protein iodinations in non-thyroid and non-tissue control systems except in one case: it definitely stimulated iodoprotein formation in homogenates of lactating mammary tissue. This finding is consistent with the observation that milk obtained from ^{131}I-treated rats contains ^{131}I-labeled protein[4].

Our studies with quinones were prompted by recent speculation on the role of vitamin K in oxidative metabolism[5,6] and by work dealing with the isolation of benzoquinones of the "Coenzyme Q" group from beef- and horse-heart mitochondria[7,8]. As shown in Table II, in addition to menadione, four naphthoquinones and one benzoquinone increased iodoprotein formation. Vitamin K_1 was not active. 2 Naphthoquinones were not only inactive in promoting iodination but actually inhibited the cytochrome *c*-activated preparations. These latter two, phthiocol and lapachol, also inhibited $^{131}I^-$-utilization by surviving slices of sheep-thyroid tissue at concentrations as low as 10^{-4} M. The iodoprotein formation promoted by the quinones was not observed when non-thyroid tissues or purified proteins were incubated in place of the thyroid tissue preparations.

TABLE II

EFFECTS OF VARIOUS QUINONES ON [^{131}I]PROTEIN FORMATION BY SHEEP THYROID WHOLE-TISSUE HOMOGENATES

(2 ml 16% homogenate incubated 2 h at 37° with 10–20 μC ^{131}I. All compounds added at $5 \cdot 10^{-4}$ M concentrations).

Compound added	*% total ^{131}I incorporated into protein*
None	2.0
Menadione	17.4
Vitamin K_1	2.7
Juglone	27.6
1,2-naphthoquinone	24.2
1,4-naphthoquinone	21.5
Lapachol	0.8
Lawsone	0.4
Phthiocol	0.2
p-benzoquinone	52.5

This work was aided by a grant from the U.S. Public Health Service.

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Received August 31st, 1959

Biochim. Biophys. Acta, 37 (1960) 189–191

The production of L-α-glycerophosphate during anaerobic glycolysis in normal and malignant tissues

Investigations of aerobic and anaerobic glycolysis in vertebrate tissues have usually assumed that the reduction of dihydroxyacetone phosphate to α-GP, catalyzed by the DPN-linked α-glycerophosphate dehydrogenase, is quantitatively a minor pathway of carbohydrate metabolism. Studies on the relative activities of glycerophosphate and lactate dehydrogenases in normal and tumor tissues[1–3] led us to reinvestigate this question since, in most tumor tissues, the levels of glycerophosphate dehydrogenase were found to be negligible in comparison to lactate dehydrogenase, while in normal tissues the levels of these two enzymes were of the same order.

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; α-GP, L-α-glycerophosphate; LA, lactic acid; HDP, hexose diphosphate.

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Kinetic and equilibrium constants of the two dehydrogenases do not significantly favor either system[3] and are compatible with a competition of the two for the electron pair produced by the oxidation of glyceraldehyde-3-phosphate. It has already been shown that the endogenous levels of the reduction products of the reactions, *i.e.*, α-GP and LA, follows the patterns of the enzyme concentration in normal and malignant tissues[4].

The data in Figs. 1 and 2 demonstrate that during anaerobic glycolysis in homogenates and in whole tissues of normal and malignant origin the formation of α-GP and LA also follows the pattern of the enzyme concentrations in these tissues.

Fig. 1 illustrates the results obtained with 600 × *g* supernatant fluids of homogenates of normal rat liver and Novikoff rat hepatoma. The animals were starved for 18 h prior to killing to reduce the level of endogenous products to negligible values.

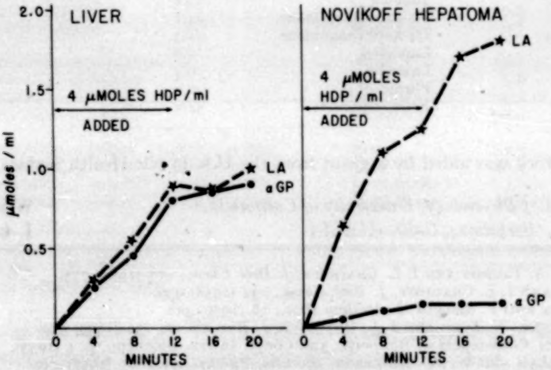

Fig. 1. Anaerobic glycolysis of homogenates. 20 % liver and 40 % Novikoff-hepatoma homogenates (equal dry weights) prepared in 1.15 % KCl containing 0.01 *M* nicotinamide and centrifuged at 600 × *g*. Reaction mixture contained 21 ml homogenate, 840 μmoles K phosphate buffer, pH 7.4, 280 μmoles $MgCl_2$; 28 μmoles $NaHCO_3$; 700 μmoles nicotinamide; 14 μmoles DPN; 140 μmoles ADP; 70 μmoles glutathione; made isotonic with KCl in a total vol. of 42 ml. 2 ml 0.084 *M* HDP was then added at constant rate over a 12-min period. 95 % N_2–5 % CO_2 bubbled through continuously; temp., 30°. 2-ml samples withdrawn at stated times; deproteinized with 0.1 ml 4.8 *M* trichloroacetic acid and assayed for LA[5] and for α-GP by a modification of the method of BUBLITZ AND KENNEDY[6].

The reaction mixture was kept anaerobic by bubbling 95 % N_2 and 5 % CO_2 through each tube. HDP was added with a constant-flow syringe over a 12-min period to avoid overloading the system with substrate, as indicated by the absence of excess HDP during the whole run. The results clearly show that in liver homogenates, glycerophosphate dehydrogenase successfully competes with lactate dehydrogenase for DPNH and three-carbon units produced during glycolysis while in hepatoma homogenates, because of drastically lowered activity of glycerophosphate dehydrogenase, little α-GP is produced.

The possibility remains that homogenization of tissues which destroys structural relationships of the enzymes of the Embden-Meyerhof pathway, may favor either α-GP or LA production. Animals were killed by decapitation and the tissues frozen either immediately or at stated intervals thereafter in order to measure formation of LA and α-GP from endogenous substrates *in situ*[7]. While normal rat liver contains sufficient endogenous substrate the tumor does not contain enough to yield measurable products of glycolysis *in situ*. The ascites form of the Novikoff hepatoma was, therefore, chosen for the tumor experiment in Fig. 2 and glucose was injected into the peritoneum at the time of killing the animals. Tumor cells were withdrawn at the stated times, homogenized in trichloroacetic acid and analyzed for α-GP and LA.

The experiments *in situ* also suggest that α-GP metabolism is an important part of carbohydrate metabolism in normal tissue and that the altered ratio of activities of the two dehydrogenases in the malignant tissue has a profound influence on the end products of anaerobic glycolysis. Essentially similar data have been obtained with a series of other normal and malignant tissues which will be reported later in a detailed publication.

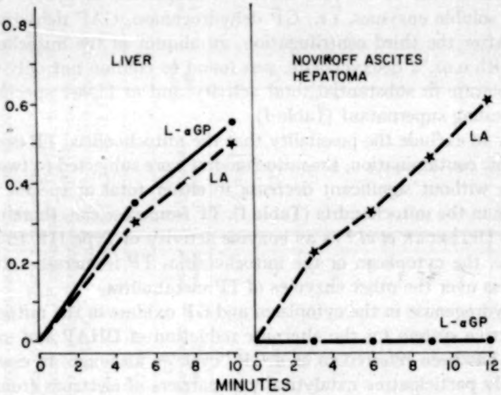

Fig. 2. Anaerobic glycolysis *in situ*. Experimental conditions described in text. Assays as in Fig. 1.

This work was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute, under the National Institutes of Health, Contract Number SA-43-ph-1886.

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Received October 15th, 1959

Mitochondrial triose phosphate isomerase

In recent studies[1–3] on the metabolism of triose phosphates in normal and malignant tissues, the importance of the intramitochondrial oxidation of GP to DHAP has been emphasized. To date it has been assumed that the product of the mitochondrial GP oxidase reentered the cytoplasmic glycolytic system at the level of DHAP. In an investigation of the distribution of TP isomerase, together with other enzymes involved in the metabolism of TP, it was found that mitochondria from rat liver and kidney retain substantial TP isomerase activity even after extensive washing.

Tissue homogenates were prepared in 0.25 *M* sucrose and centrifuged at 600 × *g* to remove nuclei and cell debris. Mitochondria were sedimented at 8000 × *g* and washed with the isolation medium as indicated in Table I. Activities of all enzymes are reported in identical units, namely mμmoles of substrate utilized per minute.

The supernatants of the first three centrifugations at 8000 × *g* contained the bulk of the soluble enzymes, *i.e.*, GP dehydrogenase, GAP dehydrogenase and TP isomerase. After the third centrifugation, an aliquot of the mitochondrial fraction, after lysis with 0.01 % deoxycholate, was found to contain not only GP oxidase but also TP isomerase in substantial total activity and at higher specific activity than the corresponding supernatant (Table I).

In order to exclude the possibility that the mitochondrial TP isomerase was due to cytoplasmic contamination, the mitochondria were subjected to two more washings with sucrose without significant decrease in either total or specific activity of the TP isomerase in the mitochondria (Table I). TP isomerase can, therefore, be classified according to DELBRUCK *et al.*[4] as an enzyme activity of Type III. In either cell compartment, *i.e.* the cytoplasm or the mitochondria, TP isomerase activity is present in large excess over the other enzymes of TP metabolism.

GP dehydrogenase in the cytoplasm and GP oxidase in the mitochondria constitute an effective system for the alternate reduction of DHAP and oxidation of GP. This system has been referred to as a GP "cycle"[1], although, in essence, DHAP or GP are simply participating catalytically as carriers of electrons from DPNH in the cytoplasm to the electron-transport chain in the mitochondria, and not in a "metabolic cycle" as usually defined. This is analogous to the function of β-hydroxybutyrate or acetoacetate described by DEVLIN AND BEDELL[5].

The presence in mitochondria of TP isomerase but not of GAP dehydrogenase does, however, permit a cyclic path for DHAP via the following series of reactions. (1) Cytoplasmic DHAP would be reduced to GP by DPNH formed on oxidation of GAP; (2) entering the mitochondria GP would be oxidized to DHAP yielding two reducing equivalents to the electron-transport chain; (3) DHAP would be isomerized intramitochondrially to GAP; and (4) GAP after transfer to the cytoplasm would again yield electrons for the reduction of *another* molecule of DHAP. The distribution of enzyme activities within the cell reported here is compatible with such a cyclic by-path at the TP level and studies to demonstrate it dynamically are now in progress.

Abbreviations: TP, triose phosphates; GP, L-α-glycerophosphate; DHAP, dihydroxyacetone phosphate; GAP, D-glyceraldehyde-3-phosphate; DPNH, reduced diphosphopyridine dinucleotide; EDTA, ethylenediaminetetraacetic acid.

TABLE I

DISTRIBUTION OF ENZYMES ACTING ON TRIOSEPHOSPHATES

Preparation of fractions and definition of units described in text. Data reported per g of wet weight of original tissue. Protein by biuret. All assays carried out under the specified conditions at pH 7.6 and 26°. TP isomerase[6]: DPNH, 0.04 mM; GP dehydrogenase, 500 units; KCN, 1.5 mM; GAP, 0.6 mM; EDTA, 5.6 mM; triethanolamine buffer. GP dehydrogenase[7]: DPNH, 0.04 mM; DHAP, 1 mM; EDTA, 5.6 mM; triethanolamine buffer. GP oxidase[8]: cytochrome *c*, 0.04 mM; KCN, 1.5 mM; GP, 6.3 mM; tris(hydroxymethyl)aminomethane buffer. GAP dehydrogenase[9]: DPN, 0.8 mM; arsenate, 13 mM; cysteine, 3.1 mM; GAP, 0.6 mM; EDTA, 5.6 mM; triethanolamine buffer.

Number of centrifugations at 8,000 × g	*Fraction*	*Liver*					*Kidney*				
		Protein mg/g	*TP isomerase units/g*	*GP dehydrogenase units/g*	*GP oxidase units/g*	*GAP dehydrogenase units/g*	*Protein mg/g*	*TP isomerase units/g*	*GP dehydrogenase units/g*	*GP oxidase units/g*	*GAP dehydrogenase units/g*
1	Supernatant	100.0	561,000	33,000	0	66,000	80.5	601,000	11,400	0	70,000
2	Supernatant	9.2	5,400	175	0	515	7.5	4,470	104	0	208
3	Supernatant	3.1	139	0	0	0	3.3	510	0	0	0
	Mitochondria	24.2	1,580	340	72	0	17.2	3,400	240	73	0
4	Supernatant	2.4	162	0	0	0	1.7	225	0	0	0
	Mitochondria	20.4	1,520	0	—	0	15.8	2,840	100	—	0
5	Supernatant	3.5	165	0	0	0	3.0	242	0	0	0
	Mitochondria	20.4	1,600	50	—	0	15.0	2,760	140	—	0

This work was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute, under the National Institutes of Health Contract Number SA-43-ph-1886.

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Received November 6th, 1959

ON THE PROPERTIES OF SOME AROMATIC RING-OPENING ENZYMES OF SPECIES OF THE GENUS *NOCARDIA*

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(Received April 8th, 1959)

SUMMARY

Oxidase enzymes which open the aromatic ring of either protocatechuic acid or catechol have been induced by growth of *Nocardia* spp. on nitrobenzoic acids. Some of their properties are described. No cofactor or metal requirements have been found for a purified, protocatechuic acid oxidase from *N. erythropolis* but, unlike the corresponding enzymes from *Neurospora* and *Pseudomonas*, the enzyme exhibits a marked activity optimum at pH 7.5. Catechol oxidase activity in *N. opaca* is very similar in properties to that of *Pseudomonas* but crude extracts prepared by ultrasonic disintegration accumulate *cis-cis*-muconic acid which has only previously been found by using a purified preparation. Tentative evidence for a lactonic type compound between β-carboxymuconic acid and β-oxoadipic acid in extracts of *N. crythropolis* is presented.

INTRODUCTION

Protocatechuic acid oxidases which transform 3,4-dihydroxybenzoic acid to β-carboxymuconic acid have been found in strains of *Pseudomonas fluorescens*[1] and *Neurospora crassa*[2,13]. This latter compound is also a substrate for lactonising and delactonising enzymes of *Neurospora*[2]. CARTWRIGHT AND CAIN[3] reported the occurrence of similar enzymes in *Nocardia* spp., this observation providing the first case of the production of such enzymes during growth on an aromatic nitro-compound. The present communication deals with a more detailed investigation of these enzymes, in particular of a protocatechuic acid oxidase and a decarboxylase complex induced by growth on *m*- or *p*-nitrobenzoic acid, and also describes the catechol-oxidase activity of extracts of *Nocardia opaca*, induced by the corresponding *o*-isomer, which activity has similar properties to a pyrocatechase elaborated by a *Pseudomonas* sp.[4].

MATERIALS AND METHODS

Organisms

These were *Nocardia erythropolis*, *Nocardia opaca* and *Nocardia* M1 isolated by enrichment techniques and grown in bulk by the methods and using the media described by CAIN[5]; CARTWRIGHT AND CAIN[3].

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Enzyme extracts

Cell-free extracts were prepared by the alumina grinding technique[6] or by ultrasonic disintegration using a 4:1 resonant chromium plated brass transforming stub attached to the transducer head of a Mullard 60-W ultrasonic drill overdriven at approximately 20 kc/sec by a 150-W amplifier. During disintegration by this method the cell suspension was cooled in alcohol–solid CO_2 and the heat balance was such that the suspension just remained unfrozen. Crude extracts were separated by high speed centrifuging in a "Spinco" Model L preparative ultracentrifuge[3].

Oxidase activity

This was followed by either (a) standard Warburg manometric techniques for O_2 uptake or CO_2 output, the particular conditions being given in the headings of the tables of results or (b) in the case of the conversion of protocatechuic acid to β-carboxymuconic acid, by spectrophotometry, a method which STANIER AND INGRAHAM[1] felt to be superior. β-Oxoadipic acid arising from further metabolism of β-carboxymuconic acid does not interfere, having a negligible light absorption throughout the u.v. region ($\varepsilon_{max} < 50$). Protocatechuic and β-carboxymuconic acids both absorb strongly and the amounts of each in mixtures can be deduced from O.D. measurements at 270 and 290 mμ[1]; the extinction coefficients at these wavelengths (pH 7.0) being respectively for protocatechuic acid 2,730 and 2,890, and β-carboxymuconic acid 6,400 and 1,590. The disappearance of β-carboxymuconic acid was followed spectrophotometrically at 254 mμ (its absorption peak) and investigations for lactonic material (β-carboxymuconolactone) were made at 220–230 mμ (where it absorbs strongly) depending on the protein concentration of the cuvette contents. Measurements were made using a "Unicam" SP 500 instrument the material under test being contained in 10 mm square section silica cuvettes.

Protein estimation

The two procedures used were (a) the biuret method[7] and (b) FOLIN's method[8].

Paper electrophoresis

The chamber consisted of a "Perspex" box (40 × 20 × 5 cm) with buffer compartments (4 × 20 × 5 cm) at each end into which were sealed carbon electrodes (15 × 2 cm diam.). The protein solution was applied as a narrow transverse band to the centre of strips of Whatman 54 paper (5–7 cm wide) which were supported between the buffer compartments by three transverse "Perspex" bars. With the buffers used, a P.D. of 110 V D.C. gave satisfactory operation. Runs were carried out at room temp. with the box closed by a plate-glass lid. Protein bands were made visible by conversion of the peptide groups to chloroamide groups by 15 min exposure of the electropherograms to an atmosphere of Cl_2 (a method brought to our attention by Dr. B. SHAW of the Department of Pharmacology). Excess Cl_2 was removed either by suspending the strips in a current of air for 10–12 h, or by a more rapid method in which most of the excess Cl_2 was removed by brief exposure in SO_2, narrow strips being cut off the main strip and tested for general absence of Cl_2 by starch–KI spray. Care was necessary with this procedure to ensure that the chloroamide groups were not also destroyed and generally SO_2 treatment was limited to reduce the overall

Cl_2 content of the paper to a low level and then the last traces removed by hanging in an air current for approx. 2 h. By this method 15 μg protein could be detected.

Chromatography

All solvent proportions are by volume. Acids and lactonic compounds were run on Whatman 542 paper in (a) ethanol–conc. NH_3 soln. (sp. gr. 0.880)–H_2O (80:4:16) or (b) *tert.*-amyl alcohol–chloroform–water–formic acid (98 % w/v) (8:8:8:3)[9]. They were visualised by an indicator spray of slightly alkaline (with NH_4OH) ethanolic bromothymol blue. Unsaturated compounds were detected with 0.5 % (w/v) $KMnO_4$ in water–acetone, 50:50 (v/v). Dinitrophenylhydrazones of oxoacids were run in pentan-1-ol–propan-2-ol–conc. NH_3 soln. (sp. gr. 0.880) (65:5:30) and detected, where weak, by their property of quenching under u.v.-light.

Buffers

2-Amino-2-hydroxypropan-1,3-diol(tris)–HCl and Na_2HPO_4–KH_2PO_4 buffers were used, at concentrations and pH values mentioned in the text. For many dialysis experiments (where mentioned) 1 mmole L-cysteine was dissolved in such buffers without alterating the pH value. 0.05 *M* KCl–sodium borate–boric acid buffer was also used on a few occasions.

RESULTS

Protocatechuic acid oxidase activity in extracts of N. erythropolis *and* Nocardia *M 1*

Extracts of *N. erythropolis*, grown on *p*-nitrobenzoic acid, were found to have high oxidase activity towards protocatechuic acid. CARTWRIGHT AND CAIN[3] described some features of this reaction in crude extracts and found all the activity lay in the soluble fraction after centrifuging at 104,000 × *g* for 30–40 min. Essentially identical activity has since been found in similar extracts prepared from *Nocardia* M 1 grown on *m*-nitrobenzoate. This has prompted an attempted purification of this oxidase from *N. erythropolis* in which the activity was usually greater.

Purification of the oxidase

The procedure, summarized in Table I, was the outcome of several trial fractionations with different batches of crude extract. No difficulty with nucleic acid elimination was encountered and so treatment with protamine sulphate or ribonuclease was unnecessary (compare STANIER AND INGRAHAM[1]).

Preliminary experiments showed that treatment of preparations less pure than Fraction 5 with calcium phosphate gel[10] stored at 0–2° for 6 months brought about appreciable refinement of the oxidase. Applying the results of trial experiments on negative and positive adsorption, Fraction 5 in 0.001 *M* phosphate buffer was treated with gel for 15 min at pH 6.0 or 6.5 and elution of the active enzymes carried out at pH 7.5 by 0.1 *M* phosphate buffer, whereby a 4-fold purification of Fraction 5 (200-fold overall purification of the protocatechuic acid oxidase) was achieved. The corresponding enzymes of *Pseudomonas* and *Neurospora* were, in contrast, purified 8 and 15-fold respectively. It is interesting to note that all decarboxylase activity of fresh extracts was lost at the Fraction 3 (Table I) stage of purification, and it appeared in the protein precipitating between 0.5 and 1.0 saturation. This quite

TABLE I

PURIFICATION OF *Nocardia erythropolis* PROTOCATECHUIC ACID OXIDASE

2 g freeze-dried cells *N. erythropolis* ground by alumina.

	Activity/ml (µl/h)	*Protein/ml (mg)*	*Specific activity (µl/h/mg protein)*	*Total activity*	*% Recovery*	*Purification*
1. Supernatant centrifuged at 104,000 × *g* for 30 min	840	11.2	75	8,400		
2. 0–0.33 satn. discarded; complete saturation with solid $(NH_4)_2SO_4$; dialysed (Fraction 2)	936	4.6	204	8,500	101	2.7
3. Fraction 2 treated saturated solution neutral $(NH_4)_2SO_4$:						
P_3 (0–0.5 saturation)	720	1.68	454	7,700	92	6.0
P_4 (0.5–0.7 saturation)	60	1.57	38	—		
Each re-suspended and dialysed						
4. Fraction 3 (P_3) treated saturated solution $(NH_4)_2SO_4$:						
P_5 (0–0.33 saturation)	12	0.90	1.3	—	—	—
P_6 (0.33–0.50 saturation)	650	0.60	1,080	7,000	83	14.4
5. Fraction 4 (P_6) similarly treated						
P_7 (0.33–0.40 saturation)	100	0.16	625	1,000	75	8.4
P_8 (0.40–0.50 saturation)	540	0.40	1,350	5,400		18.0
6. P_8 re-precipitated between 0.40 and 0.50 saturation twice and then saturated 0.40–0.45; precipitate (Fraction 5) collected	425	0.11	3,740	5,000	60	50.0
7. Fraction 5 dialysed overnight against distilled water, made pH 5.5 with acetate buffer and treated with $Ca_3(PO_4)_2$ gel (18.4 mg dry wt./ml) for 15 min. Gel: protein ratio = 10.4 : 1. The gel collected by centrifuging, washed with water, then 0.001 *M* phosphate buffer, pH 7.5; the oxidase was eluted with 0.1 *M* buffer pH 7.5 (Fraction 6)	298	0.02	15,000	840	10	200

distinct separation of oxidase and decarboxylase activity in *Nocardia* contrasts with the *Pseudomonas* system where, for resolution, gel treatment was necessary and with the *Neurospora* system where even extensive gel treatment failed to free the oxidase entirely from the decarboxylating system. All attempts to crystallize our enzyme preparations failed and it was therefore deemed necessary to investigate its homogeneity.

In electrophoresis experiments several buffers were tried, the most effective resolution and migration being achieved during 48 h at 8 mA in 0.067 *M* phosphate buffer at pH 6.0. This treatment resulted in a rapidly migrating band A (towards the anode) just leaving the strips: a narrow well-defined band, B, about 3.0 cm behind A and considerable material, C, left at the origin. In a typical experiment using 0.1 mg protein the bands were detected by cutting off a thin marker strip which was developed by the Cl_2-starch KI technique and the corresponding areas of the remaining paper cut out, eluted with 2–5 ml water, and the eluates freeze-dried to a volume of 0.1–0.2 ml, the oxidase activity then being determined spectrophotometrically. Conditions for complete elution of the protein could not be found and so exact assessment of purity was impossible, but it is unlikely that a 2-fold purification was exceeded, Table II.

TABLE II

PROTOCATECHUIC ACID OXIDASE PURIFICATION BY ELECTROPHORESIS

Experimental conditions described in the text. The assay system contained: 1.5 µmoles Na protocatechuate; 0.2 ml eluted protein; 0.067 *M* phosphate buffer, pH 7.0, 1.5 ml; water to 3 ml. The blank cuvette contained no substrate. Protocatechuate disappearance was followed at 290 mµ for 5 min. 0.10 mg protein applied to the electropherogram. (Specific activity is expressed in µl O_2/h/mg protein for comparison with Table I).

Band	*Protein (µg) eluted from paper*	*µmoles protocatechuate disappeared/5 min*	*Specific activity* $\times 10^{-4}$
A	7	0	0
B	12	1.20	27
C	27	0.40	4
Original material	20	1.12	15

In view of reported cases of apparently homogeneous fractions from electrophoresis being further resolved in an ultracentrifuge the eluate of band B cannot be categorically regarded as a solution of pure enzyme because an analytical ultracentrifuge was not available to check this. Nevertheless this eluate represents about 400-fold purification of the protocatechuic acid oxidase. Fig. 1 shows the spectrum of gel-treated oxidase (purified 200-fold). The weak peaks at 410 and 520 mµ, characteristic of a haem spectrum, were but little altered by the addition of dithionite. The spectrum shows no evidence of the presence of flavins.

Identification of the end-product of protocatechuic acid oxidation by the purified oxidase

Table III shows the stoichiometry of the reaction mediated by oxidase purified to Fraction 5 and that 2 atoms of oxygen/mole of protocatechuate are used with effectively no CO_2 evolution. The product was identified as follows:

Spectrophotometric method: McDONALD, STANIER AND INGRAHAM[11] and GROSS

TABLE III

STOICHIOMETRY OF PROTOCATECHUIC ACID OXIDATION BY PURIFIED OXIDASE

Each Warburg flask contained: KOH or H_2SO_4, 0.2 ml; 0.1 *M* phosphate buffer, pH 7.0, 1.5 ml; Na protocatechuate 1–5 µmoles; enzyme, 110 µg protein; water to a final vol. 3.0 ml. Temp. 30°; gas phase, air.

Substrate (µmoles)	*µl gas change*			*Moles/mole protocatechuate*	
	O_2	*CO_2*	*RQ*	*O_2*	*CO_2*
1	23	1	0.04	1.01	0.04
2	44	3	0.07	0.98	0.07
4	89	3	0.03	1.00	0.03
5	110	6	0.05	0.98	0.05

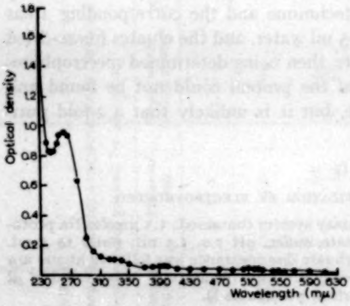


Fig. 1.

Fig. 1. The spectrum of protocatechuic acid oxidase. The sample used for this determination was the 200-fold purified product after gel treatment and concentration by lyophilization to 0.21 mg protein/ml (FOLIN method). The very weak peaks at 410 mµ, 520 mµ, 550 mµ, were unchanged in intensity by the addition of sodium dithionite.

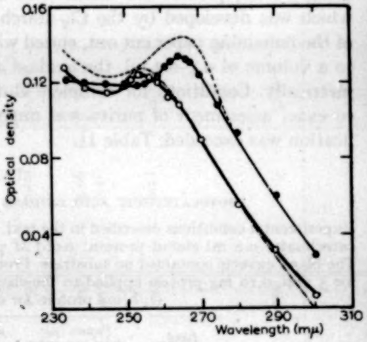


Fig. 2.

Fig. 2. The end-product of protocatechuic acid oxidation by partially purified oxidase. Conditions as for Fig. 3. At the termination of the reaction, the u.v. spectrum of the cuvette contents was measured. The spectrum of these contents after heating at 100° for 20 min, pH 7.0, was also recorded, and the spectra of authentic β-carboxymuconic acid at the same concentration before and after heating at 100° for 30 min, pH 7.0, superimposed. The results are typical of a *cis* → *trans* isomerisation. ○—○, end product of oxidation; ●—●, same after heating at 100°, 20 min; —·—·—, authentic β-carboxymuconic acid; ------, same after heating 100°, 30 min; pH 7.0.

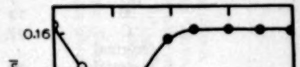


Fig. 3.

Fig. 3. The oxidation of protocatechuic acid to β-carboxymuconic acid. The reaction was followed spectrophotometrically at 270 and 290 mµ, the amounts of each acid present being calculated from the combined extinctions at the wavelengths. Each cuvette contained: 0.1 *M* phosphate buffer, pH 7.0, 1.5 ml; 1.0 ml enzyme solution, purified to stage of (P_8) Table I. The reaction was started by the addition of 0.5 µmoles (Na) protocatechuate and O.D. readings at 270 and 290 mµ made at minute intervals; 30° ± 1°. ○——○, protocatechuic acid; ●——●, β-carboxymuconic acid.

et al.[2] have shown β-carboxymuconic acid to be the reaction product of protocatechuic acid oxidases of *Pseudomonas* and *Neurospora*. Fig. 2 shows the spectra of the end-product of the action of a partially purified oxidase preparation from *N. erythropolis* and authentic β-carboxymuconic acid, together with the effects of heating on the spectra of these two materials. The conformity between the respective curves was strong evidence in favour of β-carboxymuconic acid being the product of protocatechuic acid oxidase of *Nocardia*. The shift of maximum absorption from 254 mμ to 265 mμ and increase in O.D. at the longer wavelength is typical of the *cis* to *trans* isomeric change of β-carboxymuconic acid.

Fig. 3 shows the oxidation of protocatechuic acid to β-carboxymuconic acid followed spectrophotometrically. The fact that O.D. reached and maintained a steady value indicated that the enzyme was free from decarboxylase activity. This behaviour was made the basis of tests for the presence of decarboxylase activity in other extracts.

Chromatographic method: 5 μmoles protocatechuic acid were incubated in a Warburg flask under the conditions detailed in Table III. When oxidation was complete the flask contents were passed through a column of "Amberlite" IR. 120 (H) resin (1 × 15 cm) eluted with water and the eluate reduced to a small volume (in approx. 2–3 h) over NaOH pellets in an evacuated desiccator at 0°. The solution was then applied to Whatman 542 paper together with markers of authentic β-carboxymuconic acid, and the chromatogram run in solvent system (a) for 16 h. The oxidation end-product moved a distance identical with that of β-carboxymuconic acid. A small sample of reaction mixture, and authentic β-carboxymuconic acid were separately hydrogenated by shaking with hydrogen and Adams' catalyst in Warburg flasks and the filtered reaction mixtures chromatographed as above. Two spots of identical R_F value were again obtained corresponding to β-carboxyadipic acid.

Chemical isolation: An Erlenmeyer flask containing sodium protocatechuate (352 mg) 0.1 *M* phosphate buffer pH 7.5 (250 ml) and oxidase protein (5.0 mg) were shaken at 30°, the reaction being followed by a pilot incubation in a Warburg manometer. After completion of oxidation, the product was isolated by techniques essentially similar to that used by McDONALD, STANIER AND INGRAHAM[11]. The final product (250 mg, 60 % yield) was identical in spectral and chromatographic properties with authentic samples of tri-sodium β-carboxymuconate kindly supplied by Dr. R. Y. STANIER and Dr. S. R. GROSS. Both it and authentic β-carboxymuconic acid were metabolised at the same rate by the decarboxylase enzyme fraction, and both gave 95 % yields of β-oxoadipic acid calculated by catalytic decarboxylation with 4-aminoantipyrine at pH 4.0 and 30°[12].

Some properties of protocatechuic acid oxidase

In common with the corresponding enzymes from *Neurospora*[2] and *Pseudomonas*[1] no co-factor or metal requirements were found. The 80 % reduction in activity caused by dialysis against 1 m*M* 8-hydroxyquinoline was apparently not attributable to the removal of an essential metal because addition of Fe^{++}, Fe^{+++}, Mg^{++}, Mn^{++}, Ca^{++}, Zn^{++}, Co^{++}, Ni^{++} or Cu^{++} failed to reverse the inhibition. Dialysis for up to 4 days against 1 m*M* KCN (changed daily), 1,10-phenanthroline, 2,2′-dipyridyl or Versene (EDTA) was without effect. Of several possible inhibitors tried, only sodium azide (1.5 m*M*) which caused 23 % inhibition and *p*-chloromercuribenzoate (0.01 m*M*) which was totally inhibitory had any significant effect. The enzyme activity appeared to be linked with thiol groups because the inhibition produced by 0.01 mM p-chloromercuribenzoate was completely reversed in the presence of, for example, 1 mM L-cysteine or reduced glutathione. OTTEY AND TATUM[13], however, later found a thiol and Fe^{++} requirement for this enzyme from the same *Neurospora* strain as used in[2].

Our oxidase did not attack any substrate other than protocatechuic acid. The 2,4-, 2,5-, and 3,5-dihydroxybenzoic acids were not oxidised but, like catechol, acted in different degrees as competitive inhibitors. Whereas the protocatechuic acid oxidase of *Pseudomonsa* and *Neurospora* showed increasing activity as the pH was raised from 6.0 to 9.0, the *Nocardia* enzyme had a pronounced activity peak at pH 7.5 and activity was effectively zero at pH 6.0 and 8.5 (Fig. 4). Oxygen alone acted as electron acceptor in oxidation by the *Nocardia* oxidase, ferricyanide, 2,6-dichlorophenol-indophenol and methylene blue failing to function under anaerobic conditions. The essentially irreversible nature of the reaction mediated by the *Nocardia* oxidase was shown by accumulation of the theoretical yields of β-carboxymuconate from protocatechuate. The MICHAELIS constant for the gel-treated enzyme (Fraction 6) from a LINEWEAVER-BURK plot (Fig. 5) of the effect of substrate upon reaction rate was $1.27 \cdot 10^{-3}$ M with respect to protocatechuate with a maximum velocity = $2.1 \cdot 10^{3}$ µl O_2/h.

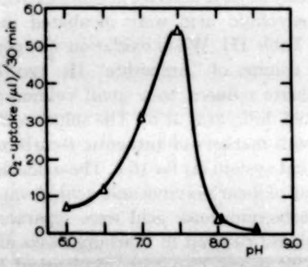


Fig. 4. The effect of pH on the oxidation of protocatechuate by partially purified protocatechuic acid oxidase from *Nocardia*. Each Warburg flask contained: KOH, 0.2 ml; Na protocatechuate, 3 µmoles; Fraction 4 (P_6), equivalent to 1.1 mg protein; buffer, 1.5 ml; total vol. 2.25 ml; air; 30°. Oxygen uptake was measured from 5–35 min after tipping the substrate. There was no O_2 uptake in the absence of substrate. △—△, 0.067 M phosphate buffer; ▲—▲, KCl + borate buffer.

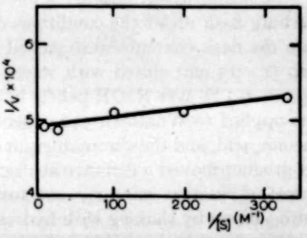


Fig. 5. The MICHAELIS constant for protocatechuic acid oxidase with respect to protocatechuate. Each Warburg flask contained: KOH, 0.2 ml; substrate, 3–50 µmoles; 0.067 M phosphate buffer, pH 7.2, 1.5 ml; Fraction 6 (= 0.16 mg protein); water to 3.0 ml. The oxygen uptake was measured from 0–15 min. There was no O_2 uptake in the absence of substrate. The velocity of the reaction (v) was taken as µl O_2/h consumed.

The nature of the decarboxylase enzyme complex

Crude cell-free extracts of *N. erythropolis* clarified by ultracentrifuging at 104,000 × g rapidly attacked β-carboxymuconic acid with the release of CO_2 and the accumulation of β-oxoadipic acid in yields of over 90 % of the theoretical. The course of metabolism of β-carboxymuconic acid by cell-free extracts is shown in Fig. 6. A sample of β-carboxymuconolactone (kindly supplied by Dr. S. GROSS) was not attacked by such extracts. The reason for this could not be specified, but the specimen was over 2 years old and may have changed to the inactive isomer. Since we had no culture of his particular strain of *Neurospora*, this fact could not be verified. Various buffers and the addition of Mn^{++}, found stimulatory for the metabolism of muconolactone by benzoate-grown cells of *Ps. fluorescens*[14] were ineffective in promoting attack on the lactone.

After the action of crude extracts on β-carboxymuconic acid, material remained which gave a positive Rothera test; this was identified chromatographically by its dinitrophenylhydrazone as β-oxoadipic acid and estimated manometrically by catalytic decarboxylation (Table IV).

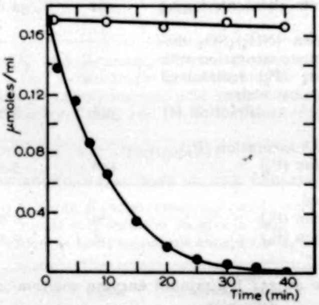


Fig. 6. The metabolism of β-carboxymuconic acid and β-carboxymuconolactone by extracts of *N. erythropolis*. The reaction was followed spectrophotometrically at 254 mμ (acid) and 230 mμ (lactone). Each cuvette contained: 0.067 *M* phosphate buffer, pH 7.0, 1.3 ml; 0.5 μmoles substrate added to initiate the reaction; 0.5 ml enzyme (crude extract dil. 1/20 = 0.13 mg protein); 0.5 ml water, or $MnSO_4$ to 1 m*M* final concentration. Quantities of remaining acid calculated from the extinction at 254 mμ (ε = 7,200). ○——○, lactone; ●——●, β-carboxymuconic acid.

TABLE IV

THE CONVERSION OF β-CARBOXYMUCONIC ACID TO β-OXOADIPIC BY CRUDE EXTRACTS

Cuvette contents as for Fig. 6, but differing amounts of β-carboxymuconic acid added. At the completion of the reaction, the cuvette contents were tipped into Warburg flasks, made acid (pH 4.0) with acetic acid + Na acetate buffer, and oxoadipic acid estimated by catalytic decarboxylation with 0.4 ml 0.1 *M* 4-aminoantipyrine added from the sidearm. The control flask contained the contents of the blank cuvette.

β-Carboxymuconic acid disappeared (μmoles)	*β-Oxoadipic acid produced (μmoles)*
0	0
0.50	0.49
1.00	0.92
1.50	1.48
2.00	1.91

Purification of the decarboxylase fraction

All decarboxylase activity lay in the protein precipitated from extracts by the addition of $(NH_4)_2SO_4$ between 0.5 and 1.0 saturation (Fraction 2 (P4), Table I).

Partial purification of this fraction was achieved by following the scheme shown in Table V.

TABLE V

THE PURIFICATION OF THE DECARBOXYLASE FRACTION

1 g freeze-dried cells irradiated 20 min, 20 kc/sec, 0–2°, and extracted with 0.05 *M* tris buffer, pH 7.2. Centrifuged to remove cell debris; cell extract ultracentrifuged.

Day of treatment	*Fraction*	*Activity**	*Protein (mg/ml)*	*Specific activity*	*Purification*
1st day	1. Supernatant from ultracentrifuging	162	12.8	12.6	·
1st day	2. 0–0.33 saturation $(NH_4)_2SO_4$ discarded; to complete saturation with solid $(NH_4)_2SO_4$ (P_1) redissolved and dialysed against water (Fraction II)	328	8.7	37.8	3.0
2nd day	3. Fraction II 0–0.5 saturation (P_2)	—	—	—	—
	0.5–1.0 saturation (P_3)	440	5.5	80	6.3
4th day	4. P_3 refractionated:				
	0.5–0.7 saturation (P_4)	70	0.84	83	6.6
	0.7–1.0 saturation (P_5)	8	0.53	15.1	·

* Activity = O.D. change at 254 mμ/min/ml enzyme solution × 10^3, measured over the interval 1–5 min.

Despite a 6.5-fold decrease in protein at stage (4), observed in all batches of extract so treated, there was only a very small increase in activity. Tests showed that the half-life of the enzyme activity was short, being about 40 h at 10° in 0.05 *M* tris buffer, pH 7.2.

The possibility existed that the loss of activity was due to oxidation of thiol groups and accordingly the effects of thiol compounds and thiol group inhibitors were investigated. Because of the marked stimulation of activity given in the presence of L-cysteine and glutathione all subsequent enzyme preparations were made in the presence of 1 m*M* cysteine and dialysed against buffers containing 1 m*M* cysteine. This resulted in retention of enzyme activity for twice as long. However, there was neither accumulation of β-carboxymuconolactone as shown by the absence of material absorbing at 220 mμ nor was there any stimulation of attack on the authentic lactone.

Decarboxylation of β-carboxymuconic acid by crude extracts was demonstrated by manometric techniques (Table VI) which showed that 1 mole CO_2 and 1 mole of β-oxoadipic acid arose from 1 mole of substrate. This reaction was not stimulated by pyridoxine, pyridoxal or diphosphothiamine at 1 m*M*.

The effect of metals and chelating agents on the decarboxylase complex

Like the lactonising-delactonising complex involved in the conversion of *cis-cis*-muconic acid to β-oxoadipic acid by *Pseudomonas*[4,14] the decarboxylase complex of *N. erythropolis* was stimulated by the addition of 1 m*M* Co^{++}, Mg^{++} and Mn^{++} (Table VII). This metal requirement was confirmed by a study of the effects of chelating agents in which the complete reversal of EDTA inhibition by Co^{++} could

TABLE VI

DECARBOXYLATION OF β-CARBOXYMUCONIC ACID BY A PARTIALLY PURIFIED ENZYME FROM *N. erythropolis*

Each flask contained: 2 N H_2SO_4, 0.2 ml; β-carboxymuconic acid, 3–5 μmoles; $MgSO_4$, 10 μmoles; cysteine, 1 mmole; 0.067 M phosphate buffer, pH 6.5, 2.0 ml; enzyme protein, 0.95 mg; water to 3.0 ml. Gas phase, O_2-free nitrogen; temp. 30°.

Substrate added (μmoles)	*CO_2 released (μmoles)*	*β-Oxoadipate formed (μmoles)*
3	2.7	2.6
4	3.7	3.6
5	4.6	4.3

Theory requires 1 mole CO_2 and β-oxoadipate/mole β-carboxymuconic acid consumed. The slightly low figures for CO_2 and β-oxoadipate are attributed to the presence of small amounts of the inactive isomer in the β-carboxymuconic acid samples received from Dr. R. Y. STANIER (University of California, Berkeley, Calif.) and Dr. S. R. GROSS (Stanford University, Calif.).

TABLE VII

THE EFFECT OF ADDED METAL IONS ON THE DECARBOXYLASE ACTIVITY

Each cuvette contained: 0.5 μmole β-carboxymuconic acid; 0.55 mg enzyme protein; 1.7 ml 0.05 M tris buffer, pH 7.2; metal salt solution to give a final concn. 1 mM; water to 3.0 ml. The cations were added as their sulphates except $CoCl_2 \cdot 6H_2O$; $CaCl_2$ (anhyd.).

Cation	*Activity, % activity in absence of added cation*
Co^{++}	110
Mg^{++}	51
Ca^{++}	18
Mn^{++}	12
Fe^{++}	0
Zn^{++}	1

be shown. The marked stimulation by Co^{++} cannot be attributed to the Cl′ ion as all experiments were performed in tris–HCl buffer and, in addition, 1 mM KCl gave no stimulation.

The participation of a lactone in the degradation of β-carboxymuconic acid

The failure of crude and partially purified extracts of *N. erythropolis* to attack a sample of β-carboxymuconolactone has already been mentioned. However, some further resolution of the enzyme complex was achieved by gel treatment and experiments with the purer material provided evidence, which was not completely conclusive, of the occurrence of a substance behaving spectrographically and chromatographically like the lactone reported by GROSS *et al.*[2]. A decarboxylase preparation (precipitated between 0.5 and 0.7 saturation with $(NH_4)_2SO_4$) was dialysed against 1 mM cysteine solution made to pH 7.0 and treated with calcium phosphate gel (gel/protein ratio = 6:1) at pH 6.4 and the supernatant subjected to similar treatment (gel/protein ratio = 10:1) at pH 6.2; the combined gels were eluted with 0.067 M phosphate buffer at pH 7.2. All activity to β-carboxymuconic acid was present in the eluate, which, despite its usual low specific activity at this stage of treatment

(O.D. decrement at 254 mμ/min/mg protein × 10^3 = 1.8), was used for detection of product absorbing at 220 mμ. The enzyme solution was placed in a cuvette with 0.5 μmole β-carboxymuconic acid and the slow disappearance of the substrate followed by reading O.D. decrements at 254 mμ, and 220 mμ. After 135 min the reaction was stopped by heating to 100° for 2 min and the u.v. spectrum of the reaction mixture read (Fig. 7). The small peak at 218 mμ is not present in the spectrum of β-carboxymuconic acid but β-carboxymuconolactone exhibits a peak at 216–219 mμ (see also GROSS *et al.*[2]). The far u.v. spectrum of the acid is not changed by the heat treatment given to stop the reaction.

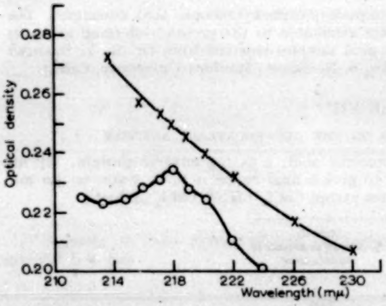


Fig. 7. The far ultraviolet spectra of authentic β-carboxymuconic acid and the reaction mixture at 135 min. The reaction cuvette contained: β-carboxymuconic acid, 0.5 μmoles; 0.067 *M* phosphate buffer, pH 6.5, 2.0 ml; enzyme, 0.5 ml. [The enzyme was Fraction 11, P_3 (Table V) treated with calcium phosphate gel and eluted with phosphate buffer at pH 7.2. Its specific activity was low (1.8)]. The reaction was stopped at 135 min by placing in a steam bath for 2 min; the spectra of reaction product and authentic β-carboxymuconic acid (0.04 μmoles) then measured. Heating has little effect upon the far u.v. spectrum (200–220 mμ) of β-carboxymuconic acid although the peak at 254 mμ shifts on heating. ×——×, β-carboxymuconic acid 0.04 μmoles/ml; ○——○, reaction mixture at 135 min.

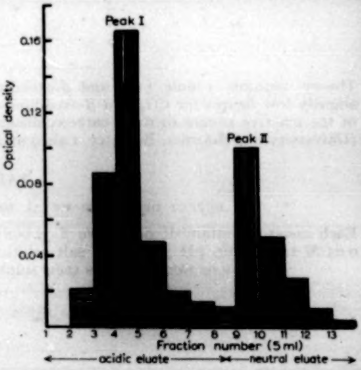
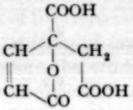


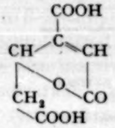
Fig. 8. The separation of a 220 mμ-absorbing compound on ion-exchange resin. The reaction mixture at 135 min from Fig. 7 was applied to an Amberlite IR 120 (H) resin column, and the components eluted with water (details in text). Peak II contains essentially only the 220 mμ-absorbing compound. ▥, absorption at 220 mμ; ▒, absorption at 254 mμ; ■, absorption at both 220 mμ and 254 mμ.

This reaction mixture was then applied to a 1 × 15 cm column of "Amberlite" IR 120 (H) resin and elution performed with distilled water. Fractions (5 ml each) were collected and examined for absorption at 220 and 254 mμ. Fig. 8 shows the O.D. of the fractions. Material absorbing at 254 mμ was present whilst the eluate was still acid but absent when the eluate ran neutral, whereas 220 mμ-absorbing material was present in both acid and neutral eluates and concentrated in tubes 2, 3, 8 and 9. The tubes containing strongly absorbing material were evaporated to dryness overnight at 0° in a desiccator, and the residues applied to papers for chromatography which was performed in solvent 1. β-Carboxymuconic acid, and its lactone, both being acidic, and unsaturated, were detectable with either the indicator or permanganate spray. Peak I contained principally material equivalent in position on the chromatograms to the acid (which also absorbs at 220 mμ) and peak II mainly material corresponding to the lactone with traces of acid. Authentic lactone and acid could be differentiated, as were the substances from the reaction mixture, by the slower bleaching of permanganate by the lactone, as well as by their respective R_F values.

The weakness of the absorption at 220 mμ and corresponding chromatographic spot showed that lactone was not accumulating, a fact borne out by the decrease in O.D. at 220 mμ when enzyme action was allowed to continue beyond 135 min. The facts described indicate that only partial resolution of the enzyme complex had been achieved. Our failure to resolve completely the complex has precluded studies on the isolated intermediates. β-Oxoadipic acid was present in reactions with gel-treated enzyme allowed to go to completion in 3–4 h. The fact that gel-treated preparations possessed only limited decarboxylase activity, as determined manometrically, yet metabolised β-carboxymuconic acid as rapidly as crude extracts (on an equivalent protein basis) suggests the existence of distinct lactonising and decarboxylating enzymes as have been found in extracts of *Pseudomonas fluorescens* utilising muconic acid[14] and that the decarboxylation step follows the possible lactonization. It is possible that the lactone formed by *Nocardia* is the 1:4 compound (γ-carboxy-γ-carboxymethyl-Δ^{α}-butenolide) and not the 3:6 isomer (β-carboxy-γ-carboxymethyl-Δ^{α}-butenolide) as in *Neurospora*. This would account for the failure of crude extracts of *N. erythropolis* to metabolise the authentic lactone (3:6) given by Dr. GROSS.



1:4-lactone



3:6-lactone (*Neurospora*)

Catechol oxidase activity in cell-free extracts of Nocardia opaca, *strain* O_4

The observation that dried cells of *N. opaca* oxidised anthranilic acid after a very brief lag period (5–10 min) pointed to a parallel between this organism and *Pseudomonas* species which oxidised the same substrate by an unstable enzyme system to catechol[15] which was further oxidised[14, 16] to β-oxoadipic acid via *cis-cis*-muconic acid and its lactone.

Adaptive oxidase activity towards catechol was demonstrated in both washed and dried cell preparations of *N. opaca* but the original extracts of this organism prepared either by alumina grinding or ultrasonic disintegration failed to attack the substrate[3]. Active extracts could be prepared only in the presence of cysteine suggesting a relationship between this enzyme and the previously reported pyrocatechase[17]. In view of the findings of BEHRMAN AND STANIER[18] that the oxidation of 6-hydroxynicotinic acid by extracts of *Ps. fluorescens* only occurred when the protein concentration in the extracts was high, care was taken to prepare similar concentrated extracts of *Nocardia opaca*. Fresh or freeze-dried cells were suspended in small volumes of phosphate buffer with cysteine at 10 m*M*, and subjected to disintegration at 20 kc/sec for 20–25 min with cooling by alcohol–solid CO_2. Extracts containing over 40 mg/ml protein were obtained and these rapidly oxidised catechol whereas weak extracts prepared in the absence of cysteine and later supplemented with thiols, showed but little activity.

Very concentrated extracts (40 mg protein/ml) showed high endogenous respiration, not diminished by dialysis for 10 h; but with the appropriate correction, 80 % of the theoretical oxygen uptake for the conversion of catechol to β-oxoadipic acid was observed. Theoretical oxygen consumption was observed with extracts containing 20 mg protein/ml.

The location of the oxidase activity in the soluble fraction of extracts is shown by the results given in Table VIII. No CO_2 evolution from catechol as substrate occurred with crude extracts, particulate fraction or soluble supernatant, and it was not initiated by addition of diphosphothiamine or pyridoxine. Activity towards protocatechuic acid and 3-hydroxyanthranilic acid was slight under these conditions (*cf.* CARTWRIGHT AND CAIN[3]). 2,5-Dihydroxybenzoic (gentisic) acid was not oxidised.

TABLE VIII

LOCALISATION OF THE CATECHOL OXIDASE ACTIVITY

Crude extracts in cysteine–phosphate buffer were centrifuged at 104,000 × *g* 30 min at 2°. The clear straw coloured supernatant was decanted off, the amber particle pellet washed with water, re-suspended in 1 m*M* cysteine, re-centrifuged, and the aqueous supernatant discarded; the pellet was then re-suspended in cysteine–phosphate buffer. Protein determinations were performed on each fraction. Each manometer flask contained: KOH, 0.2 ml; catechol, 2 µmoles; enzyme fraction (crude, 14.5 mg; supernatant, 6.4 mg; particle, 3.4 mg protein); 0.1 *M* phosphate buffer, 1.0 ml; water to a final vol. 3.0 ml; Temp. 30°; gas phase, air.

Enzyme fraction	*O_2 uptake (µl)/20 min*	Q_{O_2}
Crude extract	30	6.2
Particles	1	0.9
Supernatant	25	11.6
Particles + supernatant	30	9.2

Co-factor requirements of catechol oxidase from N. opaca

SUDA *et al.*[4] showed that the pyrocatechase of *Pseudomonas* required Fe^{++} and thiol groupings. Dialysis of the crude enzymes from *Nocardia opaca* against chelating agents showed a similar metal dependence. Freshly prepared extracts in cysteine–phosphate buffer pH 7.0 were centrifuged at 104,000 × *g* for 30 min and separate samples of the clear supernatant dialysed for 30 h against 0.05 *M* phosphate buffer, containing 1 m*M* cysteine, pH 7.0, alone, and also with this buffer containing one of the following: 1,10-phenanthroline, KCN, ethylenediaminetetra-acetic acid, 8-hydroxyquinoline, all at 1 m*M*. The catechol oxidase activity of the treated sample was determined (Table IX), the inference being that metal ions are required for activity. Similar results were obtained when the chelating agents were included in reaction mixtures with undialysed enzyme. Using the conditions described in the legend to Table IX, several divalent metals were added at a final concentration of 1 m*M* in the presence of EDTA. Ferrous iron was the only agent reversing the inhibition completely, the other ions being quite ineffective. No qualitative or quantitative differences were observed in the reversal of the inhibition caused by the other chelating agents.

TABLE IX

THE EFFECT OF DIALYSIS OF CATECHOL OXIDASE AGAINST CHELATING AGENTS

Each Warburg flask contained: KOH, 0.2 ml; catechol, 3 µmoles; cysteine, 1 mmole; 0.1 *M* phosphate buffer, pH 7.0, 1.3 ml; enzyme, 1 ml; water to 3.0 ml. Temp. 30°; gas phase, air. Each enzyme sample was estimated for protein after dialysis, the oxygen uptakes/h were calculated on equal protein basis.

Previously dialysed against inhibitor (1 mM)	*O_2 uptake/h/mg protein (µl)*	*% Inhibition*
Undialysed	38	—
Water	10	74
1,10-Phenanthroline	2	94
Versene (ethylenediamine tetra-acetic acid)	2	94
8-Hydroxyquinoline	0	100
KCN	2	94

The thiol requirement of the enzyme was confirmed by the complete inhibition of activity produced by 0.1 m*M* *p*-chloromercuribenzoic acid which was reversed by the addition of 10 m*M* cysteine or glutathione.

The end-product of the catechol oxidase reaction

In view of the fact that the crude enzyme oxidised catechol with the uptake of 2 atoms of oxygen/mole of substrate, and that the R.Q. was zero, both properties being characteristic of the conversion of catechol to β-oxoadipic acid, it was surprising to find a complete absence of oxoacids in the deproteinized supernatants left after oxidation. Furthermore the crude extracts were also inactive towards *cis-cis-*, *cis-trans-*, and *trans-trans-*muconic acids. These observations suggested that ultrasonic treatment destroyed the fraction of the enzyme system responsible for carrying the degradation beyond muconic acid and that this compound was accumulating. Therefore experiments were performed to demonstrate the presence of *cis-cis*-muconic acid in the reaction products and also to isolate it in a pure form.

1. The reaction mixture from the complete oxidation of 3 µmoles catechol by a crude extract in a Warburg flask, was deproteinized by adding 1 ml 10 % trichloroacetic acid and the suspension adjusted to pH 7.0 as rapidly as possible. A flask containing no substrate was similarly treated and used as a blank for u.v. spectrophotometric studies. The reaction mixture showed an intense peak at 257 mµ which shifted to 260 mµ with an increased absorption on boiling for 15–30 min, the original spectrum being identical with that of *cis-cis*-muconic acid, and the shift and intensification being characteristic of the *cis* ⟶ *trans* isomerisation (Fig. 9).

2. Similar duplicate sets of flasks were treated as in (1). Two of the supernatants were evaporated to about 0.3 ml of 0° in a desiccator. The other two supernatants were returned to Warburg flasks the side-arms of which contained a suspension of Adams' catalyst in water. After the flasks had been flushed with hydrogen for 15 min the taps were closed, the catalyst mixed and the flask shaken until hydrogen absorption ceased, when the catalyst was filtered off and the filtrates evaporated in a desiccator to about 0.3 ml.

The four samples of reduced and unreduced reaction product were then applied with authentic markers of *cis-cis*-muconic acid and adipic acid to duplicate sheets of Whatman 542 paper which were run in solvents (a) and (b) and detected separately with both sprays. The unreduced supernatants gave rise to spots corresponding with *cis-cis*-muconic acid whereas the reduced ones gave spots corresponding with adipic acid, *i.e.* the reduction product of muconic acid.

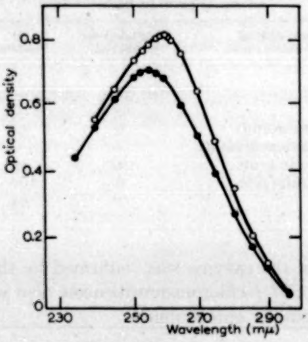


Fig. 9. Spectral identification of muconic acid as the product of catechol oxidation by crude extracts. Each Warburg flask originally contained: KOH, 0.2 ml; 0.05 *M* tris buffer, 0.6 ml; enzyme (soluble fraction of crude cell-free extract = 6.4 mg protein), 1 ml; catechol, 3 μmoles. Gas phase, air; 30°. At the completion of the oxidation, the reaction mixture was deproteinized with 0.5 ml 10% (w/v) trichloroacetic acid, and the supernatant re-made to pH 7.0 with 0.1 *N* NaOH. The spectrum of the 1/15 diluted (water) reaction mixture was then read. A sample was then boiled for 25 min and spectrum re-read. Authentic *cis-cis*-muconic acid (same concentration) gave an identical spectrum when similarly treated. In both cases there was a characteristic *cis* → *trans* shift on boiling. ●——●, initial reaction mixture; ○——○, after heating 25 min at 100°.

3. Chemical isolation of *cis-cis*-muconic acid was achieved as follows: A mixture of catechol (200 mg), 0.1 *M* phosphate buffer (70 ml), cysteine to a final concentration of 1 m*M* and extract (180 mg protein added in 2 separate lots of 110 mg and 70 mg) was shaken aerobically in a 500-ml Erlenmeyer flask until the catechol concentration was minimal (12–18 h) as deduced by $FeCl_3$ or FOLIN tests on small samples of the mixture. Deproteinization was achieved by the addition of 10% trichloroacetic acid (25 ml) and heating briefly to 70°, and after removal of the protein precipitate the supernatant was continuously extracted with ether for several hours. Acidic substances were removed from the extract by shaking twice with saturated $NaHCO_3$ solution. The aqueous layers were acidified with dil. HCl and extracted with ether (3 × 25 ml) and the extracts dried over anhydrous Na_2SO_4 and evaporated. The small residue crystallized out from a petroleum (b.p. 60–80°)–ethanol mixture to give a product, m.p. 194°, undepressed on admixture with a sample of *cis-cis*-muconic acid (m.p. 195°) prepared in this laboratory (*cf.* PARKE AND WILLIAMS[19]).

Comparison of N. opaca *catechol oxidase and previously reported pyrocatechase*

Despite the impurity of our oxidase preparations and the caution therefore necessary in drawing conclusions, the available evidence suggests that catechol oxidation by *N. opaca* follows a similar pathway to that in *Pseudomonas*[4,15] and it is brought about by a closely related enzyme system. Although extracts of *N. opaca* prepared by ultrasonic disintegration or alumina grinding failed to carry the conversion of catechol beyond *cis-cis*-muconic acid, freeze-dried preparations of the organism brought about an extensive but slow oxidation of catechol during the course of which β-oxoadipic acid, a characteristic product of the *Pseudomonas* system, was shown to be present by the Rothera test and chromatography of the 2,4-dinitrophenylhydrazones prepared from incubations. The production by our extracts of *cis-cis*-muconic acid, a substance now well established as an intermediate in the catechol to β-oxoadipic acid conversion[14, 20] was fully confirmed. Our crude oxidase was similar to that of *Pseudomonas* in its Fe^{++} and thiol requirement and pH optimum of near 7.0. Although the effects of inhibitors against the two systems differed, it is most likely that such divergences were quantitative rather than qualitative. HAYAISHI AND HASHIMOTO[17] reported that KCN, and 8-hydroxyquinoline had no effect when added to their purified enzyme and SUDA *et al.*[4] that 2,2′-dipyridyl had little effect on a partially-purified enzyme, whereas these reagents brought about almost complete inhibition with the *Nocardia* preparation. Furthermore, dialysis of the purified *Pseudomonas* enzyme had no effect on its activity yet resulted in 74% inactivation in our preparation, the activity being restored on the addition of Fe^{++} and a thiol. A possible explanation of the differences in behaviour towards inhibitors and dialysis may lie in the fact that the iron–enzyme–substrate complex of *Pseudomonas* is less readily dissociated than that of *Nocardia* (see also MASON[21]).

ACKNOWLEDGEMENTS

We wish to thank the Department of Pharmacology for allowing us the use of their ultracentrifuge and spectrophotometers and Miss J. COOPER for technical assistance. R.B.C. wishes to acknowledge the receipt of a D.S.I.R. Research Studentship during the tenure of which this work was carried out.

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CARNOSINE-ANSERINE SYNTHETASE OF MUSCLE

III. EXPERIMENTS ON THE MECHANISM OF PEPTIDE BOND FORMATION

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(Received April 4th, 1959)

SUMMARY

Studies of the mechanism of carnosine and anserine formation have been performed with an ATP-dependent enzyme from chick muscle. The process of dipeptide formation was strongly inhibited by the addition of pyrophosphate or hydroxylamine. Although several techniques were used, it was not possible to detect an activated β-alanine intermediate. Synthetic β-alanine adenylate was found to react non-enzymically with histidine and methylhistidine, and also with other types of amino acids, to form peptides. Accordingly its role in carnosine-anserine biosynthesis could not be readily ascertained.

INTRODUCTION

The numerous recent studies on enzymic activation of α-amino acids are usually interpreted by a reversible mechanism of the type:

$$\text{ATP} + \text{amino acid} \xrightleftharpoons{\text{Mg enzyme}} \text{amino acyl — AMP} + \text{PP}$$

The difficulties encountered in attempts to isolate or identify the adenyl amino acids have led to the view that these compounds do not normally exist in the free state, but are rather in a complex with the enzyme[1,2]. Despite these difficulties, at least one enzymically-synthesized amino acyl adenylate, that of tryptophan, has been isolated and well characterized[3]. Also, the experiments of HOAGLAND *et al.*[4], which demonstrated an enzyme-induced transfer of isotopic oxygen from the COOH group of an amino acid to AMP, offer striking evidence for amino acid adenylate formation.

NOVELLI[5] points out that the ratio of carboxyl group activation (measured by hydroxamic acid formation) to [^{32}P]pyrophosphate incorporation into ATP, may vary widely from unity with different amino acids and enzyme preparations, and he cautions against drawing general conclusions from the application of these techniques Nevertheless, both approaches are used extensively.

Variants of the above mechanism for amino acid activation have been suggested. Thus OGATA *et al.*[6] described a reaction between the "pH 5" enzyme system and

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The abbreviations used are: ATP and AMP-adenosine tri-, and monophosphates; RNA – ribonucleic acid; PP – inorganic pyrophosphate; Tris – tris(hydroxymethyl)aminomethane; CAS – carnosine–anserine synthetase.

β, γ-^{32}P-labeled ATP, which resulted in a transfer of radio-active PP to RNA. They suggest that the bound PP may then be substituted by amino acid. Also SNOKE AND BLOCH[7] proposed that glutathione synthesis involves phosphorylation of the enzyme as a first step.

The ATP-dependent CAS of muscle tissue provides an interesting model system for the study of β-alanyl peptide synthesis[8,9]. The mechanism of this process, not hitherto investigated in detail, forms the subject of the present paper. It has not proved feasible to study the incorporation of labeled PP into ATP with our enzyme preparation, since a very rapid reaction occurs in the absence of amino acid substrate. Nor has it been possible to use radioactive ATP (in the manner of OGATA *et al.*[6]), because of extensive breakdown of the nucleoside triphosphate (due to contaminating enzymes in CAS). Several types of experiments have been performed with [^{14}C]amino acids, to detect formation or accumulation of an activated β-alanine intermediate. Included were experiments with synthetic adenyl β-alanine, and inhibition studies with PP and with hydroxylamine.

MATERIALS AND METHODS

Isotopic animo acids

DL-[1-^{14}C]histidine, 1-[1-^{14}C]methylhistidine, and β-[1-^{14}C]alanine were preparations previously described. DL-[1-^{14}C]valine and DL-[1-^{14}C]leucine were purchased from the Radiochemical Centre, Amersham, England. All amino acids were employed at a specific activity level of the order of 0.1 mC/mmole.

β-alanine adenylate

The method of MEISTER[10] was used to synthesize this compound. The procedure involved the use of N,N′-dicyclohexylcarbodiimide to effect the condensation of the COOH of carbobenzoxy β-alanine with the phosphate of AMP in aqueous pyridine. Subsequently the carbobenzoxy group was removed by catalytic reduction. The product was finally freed of a small proportion of accompanying AMP by passage through a column of Dowex resin[1]. The ratio of nucleotide (spectrophotometric analysis) to amino acid content (ninhydrin colorimetry[11]) was 1.05. Paper chromatographic analysis revealed only traces of free β-alanine. β-alanine adenylate was also synthesized by the method of BERG[1] and found to give results virtually identical to those obtained with MEISTER's preparation, in the experiments described in the next section.

Carnosine-anserine synthethase

This was a protein fraction of chick pectoral muscle extract, precipitated between 20–60 % ethanol concentration.

Assay for dipeptide synthesis

The method was that previously described[9]. Unless otherwise stated, the reaction mixture contained per ml: 3 μmoles ATP, 2 μmoles Mg^{++}, 0.5 μmoles isotopic amino acid (generally histidine or methylhistidine), and 6 mg of CAS powder, all in 0.04 *M* pH 7.4 Tris buffer. In experiments with β-alanine-AMP, this compound was used at 2 μmoles/ml (unless otherwise specified). The usual incubation time was 1 h at 37°.

RESULTS

Comparison of β-alanine adenylate with free β-alanine plus ATP

In view of current interest in synthetic adenyl amino acids as possible intermediates in protein synthesis[12], it appeared of significance to test β-alanine-AMP in connection with dipeptide formation. Fig. 1 shows that this compound was considerably less effective than β-alanine + ATP and Mg, in both carnosine and anserine synthesis in the presence of CAS. Exact comparisons are difficult, since the reaction rate with β-alanine and ATP was linear for more than 1 h, while the reaction of the adenylate was largely completed in 10 to 15 min. Also, in the former case maximum velocity was attained at about 2 μmoles β-alanine/ml, while no such limit was reached with β-alanine-AMP over the range tested (up to 4 μmoles/ml). The increased activity of β-alanine-AMP in the presence of ATP and Mg was due very likely to the utilization of free β-alanine, released by decomposition of the adenylate during the incubation period. The bottom pair of curves in Fig. 1 shows that no activity could have resulted from the breakdown of β-alanine-AMP in the absence of ATP.

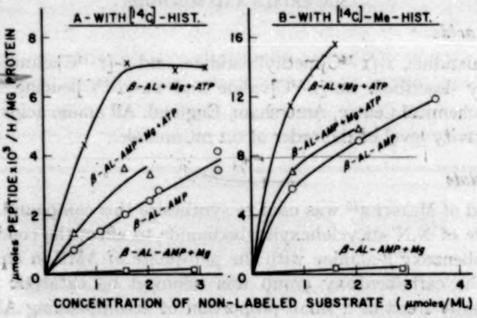

Fig. 1. Comparison of β-alanine with β-alanine adenylate under various conditions. AMP was used at 2 μmoles/ml, while ATP and Mg concentrations were as stated in the section under methods.

Since there are indications that adenyl amino acids can react spontaneously with amino groups to form peptidic structures[10], we were led to examine this possibility with β-alanine adenylate. Indeed the latter substance was found to form carnosine and anserine as readily with heat-denatured enzyme, or even in the absence of enzyme, as in the presence of CAS (Fig. 2). In fact the condensation process appeared to be non-specific, and more active with leucine and valine. On the other hand, only histidine and certain of its derivatives can be utilized by CAS in the presence of β-alanine and ATP[9]. It is curious (and may be simply a coincidence) that the non-enzymic reaction occurs more readily with methylhistidine than with histidine, just as in the enzymic synthesis with β-alanine and ATP.

It could be shown that the extent of carnosine formation with a mixture of β-alanine-AMP and β-alanine (plus ATP and Mg) was equal to the sum of the effects obtained when the 2 forms of the amino acid were tested separately (Table I). This additive result suggests that different types of reactions occurred in the 2 cases.

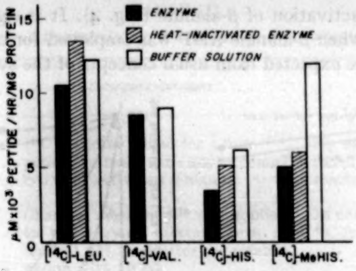

Fig. 2. Reaction of β-alanine-AMP with different amino acids under enzymic and non-enzymic conditions.

TABLE I

COMBINED EFFECT OF FREE β-ALANINE AND β-ALANINE ADENYLATE IN CARNOSINE SYNTHESIS

[^{14}C]histidine was used in each assay. Mg^{++} and ATP were added in those cases in which β-alanine was employed.

Non-labeled substrates, μmoles/ml		*Peptide synthesized, μmoles × 10³/h/mg protein*
β-alanine	*β-alanine adenylate*	
0.45	—	6.3
	1.5	5.0
—	3.6	8.8
0.45	1.5	11.6
0.45	2.4	12.8
0.45	3.6	14.2

In Fig. 3 it may be seen that the non-enzymic reaction with β-alanine-AMP took place about as readily at pH 6.5 as at 7.4. By contrast, CAS was much less active with β-alanine and ATP at the more acidic pH, in agreement with earlier findings[8].

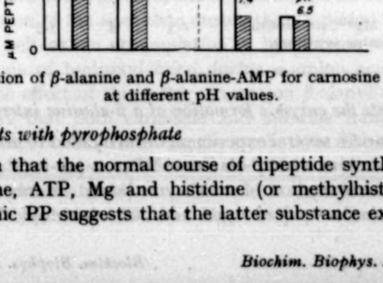

Fig. 3. Relative utilization of β-alanine and β-alanine-AMP for carnosine and anserine synthesis, at different pH values.

Inhibition experiments with pyrophosphate

The observation that the normal course of dipeptide synthesis by CAS in the presence of β-alanine, ATP, Mg and histidine (or methylhistidine) was strongly inhibited by inorganic PP suggests that the latter substance exerted a mass action effect to repress the activation of β-alanine (Fig. 4). It is significant that PP was relatively ineffective when β-alanine-AMP was employed for carnosine and anserine formation, as would be expected from usual concepts of the synthetic process.

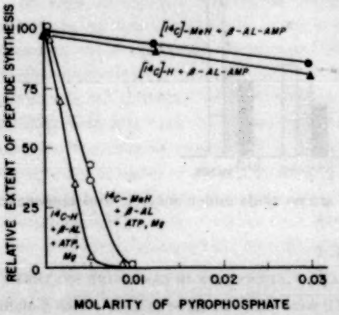

Fig. 4. Effect of pyrophosphate on dipeptide synthesis in experiments with free- and with nucleotide-bound β-alanine. The 100% values were obtained with each [^{14}C]amino acid in the absence of PP.

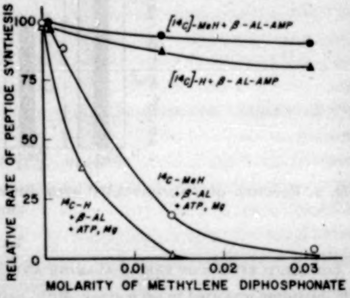

Fig. 5. Effect of methylene diphosphonate on carnosine and anserine formation. The values are refered to controls, as in Fig. 4.

The availability of a PP analog, methylene diphosphonic acid, $H_2O_3P–CH_2–PO_3H_2$, suggested its use in analogous experiments (Fig. 5). The results are similar to those with PP: the analog inhibited "normal" dipeptide formation (at slightly higher concentrations than with PP), but had no marked effect when β-alanine-AMP was employed.

Mindful of an alternative explanation, namely that PP interferes with peptide bond synthesis through combination with Mg, we performed the experiment summarized in Table II. The observation that relatively high concentrations of magnesium ions failed to overcome the inhibition which PP exerted on carnosine synthesis, makes the above possibility seem less likely, and confirmed the view that added PP acted in the manner already suggested.

TABLE II

INABILITY OF EXCESS MAGNESIUM TO REVERSE THE INHIBITORY EFFECT OF PYROPHOSPHATE

5 μmoles/ml pyrophosphate were present in each case. [^{14}C]histidine was the isotopic amino acid used. The results are in % of the control value ($9.5 \cdot 10^{-3}$ μmoles peptide/h/mg protein, with 2.5 μmoles Mg^{++}/ml and no pyrophosphate added).

Concentration of added Mg^{++}, μmoles/ml	2.5	4	5.5	7	8.5	10
Relative degree of carnosine synthesis	13	14	12	10	9	7

Attempts to demonstrate the enzymic formation of a β-alanine intermediate

Table III summarizes several experiments which failed to detect an accumulation of activated β-alanine. Also when β-alanine-AMP was pre-incubated with CAS in an attempt to induce the formation of an enzyme-bound intermediate (Table IV), no extra dipeptide synthesis resulted upon addition of isotopic histidine or methyl-

TABLE III

ATTEMPTS TO DETECT AN ACTIVATED β-ALANINE INTERMEDIATE

Components present during preliminary 1-h incubation with CAS	*Subsequent operations*	*Results*
β-alanine, Mg^{++}, ATP	$[^{14}C]$histidine added for 5-min period, to react with any accumulation of activated β-alanine	No extra carnosine formed, compared to 5 min assay with all reactants present
β-$[^{14}C]$alanine, Mg^{++}, ATP	Charcoal adsorption[13] was applied to the enzyme mixture both at neutral pH, and after deproteinization with $HClO_4$	In neither case could an adsorbable ^{14}C-fraction be detected
β-$[^{14}C]$alanine, Mg^{++}, ATP	CAS was precipitated at 0° by 70% ethanol. The filtrate was subjected to paper chromatography at 0° (*n*-butanol–formic acid–H_2O), while the protein precipitate was washed with ethanol	No β-$[^{14}C]$alanine adenylate was detected on the chromatogram, nor did the protein contain significant radioactivity. Virtually all of the ^{14}C was accounted for as free β-alanine

TABLE IV

EFFECT OF PRE-INCUBATION OF β-ALANINE ADENYLATE UPON SUBSEQUENT DIPEPTIDE SYNTHESIS

Substrate incubated with CAS for 1 h	*Subsequent additions*	*μmoles × 10³ peptide formed/mg protein in following 10 min*
β-alanine-AMP	$[^{14}C]$histidine	0.6
None	$[^{14}C]$histidine + β-alanine-AMP	3.0
β-alanine-AMP	$[^{14}C]$methylhistidine	1.6
None	$[^{14}C]$methylhistidine + β-alanine-AMP	7.8

TABLE V

INHIBITION OF SYNTHETASE ACTIVITY BY HYDROXYLAMINE

Molar concentration of salt-free NH_2OH	*μmoles × 10³ peptide synthesized/h/mg protein*	
	with $[^{14}C]$histidine	*with $[^{14}C]$methylhistidine*
0	6.5	9.8
0.6	2.0	4.3
1.0	0.8	1.9

histidine. The low values in the first and third experiments here most probably reflect extensive decomposition of the adenylate during the 1 h period at 37°.

In view of the well-known accumulation of hydroxamic acids in the presence of high concentrations of hydroxylamine during α-amino acid activation, it was important to test the effect of this latter reagent on β-alanyl peptide synthesis by CAS. Table V shows that both carnosine and anserine formation were strongly inhibited by the presence of NH_2OH during the incubations. The effects are comparable to those observed by HOAGLAND *et al.*[14] with the pH 5 enzyme system.

Salt-free hydroxylamine (1 *M*) was also used in experiments in which CAS was incubated with β-$[^{14}C]$alanine, ATP, and Mg, and paper chromatographic analysis subsequently performed, in order to detect β-alanine hydroxamate formation. However, these attempts were negative. No hydroxamate could be located by either radioactivity or by the $FeCl_3$ color test, although several solvent systems were used. These unsuccessful experiments may simply reflect the low activity of CAS, as compared with some of the enzymes that activate α-amino acids. Or alternatively, it is possible that β-alanine-AMP is not an intermediate, and that NH_2OH inhibits CAS in a different manner than via hydroxamate accumulation.

DISCUSSION

The present results indicate that CAS, β-alanine and ATP interact to activate the amino acid for peptide bond formation. While other mechanisms can be proposed, the pathway via an adenylate has not been eliminated, and will receive further consideration in future studies with purified synthetase.

Our work does suggest that the activated β-alanine intermediate will neither ordinarily accumulate, nor exist, uncombined with enzyme. Certainly if β-alanine adenylate were released to any marked extent in muscle tissue, a non-enzymic condensation with various amino acids (in addition to histidine) might be expected. Actually the process appears to be highly selective, since only L-histidine (and certain analogs) forms β-alanyl peptides. Accordingly, CAS must function *in vivo* by effecting the combination of β-alanine with its physiological acceptor.

ACKNOWLEDGEMENTS

We are obliged to Dr. A. MEISTER, Tufts University, Boston; and Dr. P. BERG, Washington University, St. Louis, U.S.A., for making available to us helpful details of their methods for synthesizing amino acyl adenylates. Our thanks are also due to Drs. H. LIS and J. J. STENESH for aid in the preparation of β-alanine adenylate and for suggestions in the course of this investigation. A sample of methylene diphosphonic acid was kindly supplied by Dr. F. KÖROSY.

This research was supported by a grant from the Muscular Dystrophy Associations of America.

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A SELF-REPRODUCING SYSTEM CONCERNED WITH THE FORMATION OF CHLOROPLASTS IN *EUGLENA GRACILIS*

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(Received April 9th, 1959)

SUMMARY

Some strains of *Euglena gracilis* can permanently lose their ability to form chloroplasts when exposed to temperatures of 34–35° under growing conditions.

If cells not yet irreversibly bleached are returned to lower temperatures, their rate of multiplication will determine whether the hereditary character will be lost or maintained. Rapidly growing cells become permanently bleached; cells dividing slowly or not at all rapidly regain their full ability to form chloroplasts.

The results indicate the existence in *Euglena* of a self-duplicating system involved in the formation of chloroplasts. This system is characterized by a replication mechanism autonomous with respect to that of the cell as a whole and by a high susceptibility to environmental factors.

INTRODUCTION

Some strains of *Euglena gracilis* lose their ability to form chloroplasts when grown either at the critical temperature range of 34–35°[1] or in the presence of streptomycin[2]. This treatment leads to permanently "bleached" but viable organisms and involves, therefore, the loss of a hereditary character from the cells.

This bleaching effect has been considered as a preferential inhibition of chloroplast multiplication, with the eventual appearance of cells without plastids[3,4]. But recent work in this laboratory on the bleaching phenomenon in *Euglena gracilis*, strain z[5], has suggested that maintenance at the bleaching temperature prevented the production of a factor required for an early step in the formation of chloroplasts. The permanent loss of the chloroplast-forming ability was explained by ascribing a self-reproducing character to the cellular system that produces this factor.

In the present study, cells at various stages of the bleaching treatment were examined for their capacity of forming green or bleached colonies. The results indicate that if cells at an advanced state of bleaching, but not yet affected irreversibly, are returned to a normal temperature, they will yield green populations when multiplying slowly, and bleached populations when engaged in rapid growth. *This dependence of the retention of the hereditary character upon the rate of cell division suggests that the self-reproducing system involved possesses a replication mechanism that is autonomous in respect to that of the cell as a whole.*

EXPERIMENTAL

Methods

The organism, culture media, and procedures for the determination of chlorophyll and cell number have been described previously[5,6].

Production of colonies

The medium for plating consisted of 1.5 % agar, 1 % proteose peptone, 0.4 % sodium acetate and 0.4 % yeast extract. Samples of the cultures were diluted in sterile distilled water to concentrations of 1000 to 4000 cells/ml, and 0.1-ml portions were either spread on freshly prepared solidified agar medium, or mixed in the Petri dishes with the agar kept liquid at 45°. The Petri dishes were kept at room temp. under constant illumination. Within 1 week, the colonies were sufficiently large to be counted. The two methods of plating yielded similar proportions of bleached and green colonies, but the second gave better recoveries (90–100 %) and more compact colonies.

Typical bleaching experiment

Normal green cells were inoculated into fresh growth medium. Usually, 1 ml of a culture at an advanced state of growth (10^6 cells/ml) was used for each 100 ml of medium. The culture was incubated at 34.5° under constant illumination. This will be referred to as the "bleaching culture". After 3 days at 34.5°, a portion of the bleaching culture was transferred aseptically to a separate flask and incubated at room temp. This will be called "recovering culture". Samples of the bleaching and recovering cultures were taken at regular time intervals (usually 24 h) for plating and chlorophyll determination. The temperature of 34.5° will be called "bleaching temperature" and the term "recovery" will apply to the course of events taking place when the cells subjected to the bleaching treatment are returned to room temperature.

RESULTS AND DISCUSSION

Behavior of chloroplasts during bleaching and recovery

It has been shown previously[5] that cells exposed to the bleaching temperature still formed chloroplasts. The increase in chloroplasts with respect to time was, however, now linear instead of exponential and the cells became progressively depleted of their chloroplasts. When portions of the bleaching culture were returned to room temp., rapid formation of chloroplasts was resumed, but only after a considerable lag period. This lag increased with the duration of exposure of the cells to the bleaching treatment. After several days of the bleaching treatment the cultures were still capable of returning their chlorophyll content to normal values at room temp., which suggested that no irreversible change had yet taken place. On the other hand, when small samples of the same cells were inoculated in fresh medium and allowed to grow at room temp., considerable proportions of completely bleached cells appeared in the subcultures[6].

Changes in the ability of bleaching and recovering cells to form green colonies

Mixtures of completely colorless and fully green colonies were obtained from most stages of bleaching and recovery. A small proportion of the colonies was usually pale green, or green in the center with colorless fringes. They were included in the green colony counts.

In Fig. 1, the proportions of green and bleached colonies obtained in agar were multiplied by the cell concentrations in the culture, in order to obtain the changes in the total number of cells yielding either green or bleached colonies. In this experiment, a small amount of cells leading to bleached colonies appeared after 2 days. As the treatment continued, the number of cells yielding bleached colonies increased rapidly and the number of cells producing green colonies decreased. After 120 h of bleaching, green colonies were no longer formed. Although varying proportions of green and bleached colonies were obtained as the treatment progressed, the cells in the bleaching culture appeared equally pigmented*.

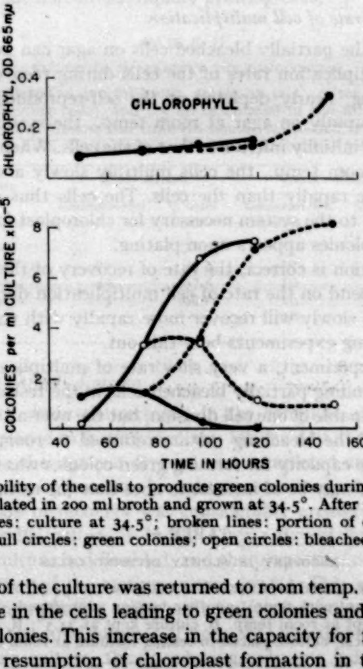

Fig. 1. Changes in the ability of the cells to produce green colonies during bleaching and recovery. 2 ml green culture inoculated in 200 ml broth and grown at 34.5°. After 74 h, 50-ml sample placed at room temp. Full lines: culture at 34.5°; broken lines: portion of culture returned to room temp.; full circles: green colonies; open circles: bleached colonies.

When a portion of the culture was returned to room temp. after 74 h of bleaching, there was a rapid rise in the cells leading to green colonies and a decrease in the cells yielding bleached colonies. This increase in the capacity for forming green colonies preceded by far the resumption of chloroplast formation in the recovering culture. After 2 days of recovery, the proportion of cells leading to green colonies had increased from 30 % to 86 %, but no change had yet taken place in the chlorophyll content of the recovering culture.

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* Recovery (return of part of the culture to room temp.) was usually started after 3 days of bleaching treatment. The proportion of green and bleached colonies obtained at this stage could be varied by slightly changing the size of the inoculum at the beginning of the experiment. The proportion of green colonies increased or decreased with the size of the inoculum. This relation is probably due to variations in the extent of cell multiplication and the corresponding dilution of the self-duplicating system at this stage of growth.

The observed shifts in the numbers of cells leading to green and bleached colonies during recovery could be explained by a rapid multiplication of the cells not yet irreversibly bleached, coupled with a preferential death of the permanently bleached organisms. But it must be understood that the recovering culture was already approaching the stationary phase of growth and could not be expected to support a rapid cell proliferation. Furthermore, the conditions during recovery are not selective against bleached cells. The observed changes may be explained better by the assumption that cells that yielded bleached colonies at the time when they were withdrawn from bleaching conditions regained the capacity to form green colonies during the recovery. This implies that cells not yet irreversibly bleached can produce bleached colonies when placed on agar at room temp.

Relation of recovery to rate of cell multiplication

The behavior of the partially bleached cells on agar can be explained by a consideration of the multiplication rates of the cells during recovery. If the cells were, at the time of plating, nearly depleted of the self-reproducing system, and then allowed to multiply rapidly on agar at room temp., the system could be lost if its rate of replication was initially inferior to that of the cells. When the bleaching culture itself is returned to room temp., the cells multiply slowly and the self-duplicating system increases more rapidly than the cells. The cells thus gradually become replenished with respect to the system necessary for chloroplast formation and a higher proportion of green colonies appears upon plating.

If this interpretation is correct, the rate of recovery of the capacity for forming green colonies will depend on the rate of cell multiplication during the recovery: the cells multiplying more slowly will recover more rapidly with respect to green colony formation. The following experiments bear this out.

In one type of experiment, a very slow rate of multiplication during recovery was achieved by suspending partially bleached cells in the resting medium[6]. The cells thus treated are still capable of one cell division, but the over-all rate of multiplication is slower than that of the bleaching culture returned to room temp. As expected, a faster recovery of the capacity for forming green colonies was obtained under these conditions (Table I). It may be of interest to note that the resting medium, although

TABLE I

RECOVERY IN SLOWLY DIVIDING CELLS

1.5 ml of green culture inoculated into 200 ml broth, grown at 34.5°. After 70 h, one portion of bleaching culture placed at room temp.; another portion centrifuged, cells washed, suspended in resting medium and kept at room temp. B, culture kept at 34.5°; R, culture placed at room temp.; RM, culture placed in resting medium at room temp.

Time h	*Chlorophyll (per cell)**			*Green colonies (% of total)*		
	B	*R*	*RM*	*B*	*R*	*RM*
70	0.50	0.50	0.50	72	72	72
95	0.36	0.26	0.28	16	95.5	99.0
119	0.36	0.54	0.28	1	97.5	99.5
142	0.30	0.96	0.32	0	99	100
167			0.30			

* Arbitrary units; a normal cell would have a value of approx. 1.0.

more favorable for a rapid recovery of the ability to form green colonies, strongly represses the resumption of chlorophyll synthesis in the recovering cells (see Table I).

In another type of experiment, the bleaching cells were made to multiply very rapidly during recovery by the inoculation of a small portion of the culture in fresh broth at room temp. (see Table II). The capacity of the culture to form green colonies decreased at first although the cells were kept at room temp. (Table II, Fig. 2). Eventually, the culture began slowly to recover that capacity concurrently with the increase in the generation time of the cells (Table II). Considerable proportions of the cells became irreversibly bleached during this rapid growth, as evidenced by the presence of many completely colorless cells among the green ones after many days at room temp. The rate of multiplication of the recovering self-duplicating system is apparently lower than that of the rapidly growing cells.

TABLE II

RECOVERY IN RAPIDLY DIVIDING CELLS

1.5 ml green culture inoculated in 200 ml broth, grown at 34.5°. After 70 h, one portion placed at room temp. (slowly dividing cells), and 0.5 ml inoculated in 200 ml broth and kept at room temp. (rapidly dividing cells). Generation time (in hours) calculated from rate of growth in the previous 24 h. % Green: green colonies as per cent of total colonies.

Time h	*Slowly dividing*		*Rapidly dividing*	
	Generation time	*% Green*	*Generation time*	*% Green*
70		72		72
95	22	95.5	10	46
119	40	97.5	10	41
142	115	99	16	53
167			18	67

Recovery in the absence of cell division

It was possible to study the recovery in the complete absence of cell division by keeping the cells, placed in resting medium, for one day at the bleaching temperature. During this time the last cell division was completed and no further multiplication occurred when the culture was brought to room temp. As can be seen in Fig. 3 and Table III, the rate of recovery was considerably greater in the resting culture as compared to a parallel culture in broth. After 1 day of recovery, the number of cells in the growing culture had doubled, and the proportion of green colonies (34 %) was about one half that of the resting culture (73 %). This would suggest that the same number of self-reproducing units had been formed in both cultures after 1 day of recovery, but that the total number of units was distributed in twice the number of cells in the growing culture, thus producing only one half the amount of green colonies. It can also be seen in Table III that the number of green colonies declined more slowly in the resting culture kept at the bleaching temperature.

Significance of plating of partially bleached Euglena *cells*

The experiments described above indicate that after a few days of bleaching treatment the hereditary character, although still present in most cells, may be lost if the cells are allowed to multiply rapidly after the treatment is interrupted. Since such rapid multiplication must occur when the cells are transferred to agar, cells

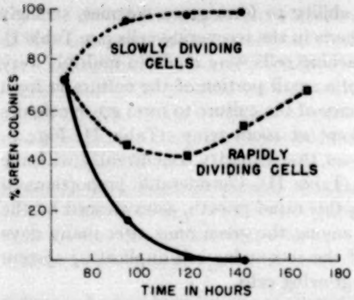

Fig. 2. Ability to form green colonies in relation to the rate of cell multiplication during recovery. Experimental conditions: see Table II. Full line: culture kept at 34.5°; broken lines: cultures at room temp.; circles: bleaching and recovering cultures (slowly dividing cells); squares: inoculum from bleaching culture in fresh broth (rapidly dividing cells).

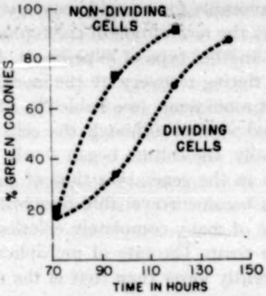

Fig. 3. Recovery in non-dividing cells. Experimental conditions: see Table III. Circles: cells recovering in broth (dividing cells); squares: cells recovering in resting medium (non-dividing cells).

not yet irreversibly bleached will yield bleached colonies. The probability that the self-duplicating system will be lost upon plating will increase with the degree of depletion of the cells with respect to this system. This situation is reflected by the proportion of green and bleached colonies obtained on plating. The plating technique, therefore, provides some information on the relative number of self-duplicating units present in the cells. This is confirmed by the results obtained with the non-dividing cells (Table III).

It is possible to estimate the number of irreversibly bleached cells at a given stage of bleaching by returning part of the culture to room temp. and allowing the recovery to proceed for a long time. The number of bleached colonies will decrease at first, but will eventually reach a constant level (Fig. 1). This final value can be

TABLE III

BLEACHING AND RECOVERY IN NON-DIVIDING CELLS

Green cells inoculated in broth to an initial concn. of 6,200 cells/ml and incubated at 34.5°. After 48 h, part of the culture collected aseptically, suspended in a smaller volume of resting medium and returned to the bleaching temperature for one more day to allow for additional cell division under bleaching conditions. At the end of this period (total bleaching time: 72 h), both the cultures in resting medium (non-dividing cells) and in growth medium (dividing cells) were divided into 2 portions. One was kept at the bleaching temperature of 34.5° (B), and the other one placed at room temp. (R).

Time h	*Dividing*				*Non-dividing*			
	Cells/ml culture $\times 10^{-3}$		*% Green colonies*		*Cells/ml culture* $\times 10^{-3}$		*% Green colonies*	
	B	*R*	*B*	*R*	*B*	*R*	*B*	*R*
72	266	266	17	17	420	420	18	18
96	290	520	0.3	34	438	445	3	73
120	308	565	0	69	418	440	2	91
142	363	630	0	87				

taken as a rough measure of the amount of bleached cells initially present. As can be seen in Table IV, the proportion of bleached colonies remaining after a prolonged recovery increases with the degree of depletion of the cells at the beginning of recovery.

A further indication that the cells giving rise to bleached colonies are not necessarily bleached irreversibly is provided in Table V. In this experiment, the cells had been subjected to a prolonged bleaching treatment and had not yielded any more green colonies for 2 days before they were returned to room temp. After 2 days of recovery, some ability to form green colonies was regained.

TABLE IV

RELATION BETWEEN EXTENT OF BLEACHING AND EXTENT OF RECOVERY

Each line represents a separate experiment. The "initial" values describe the length of exposure to the bleaching temperature and the percentage of bleached colonies obtained at the time the cultures are returned to room temp. The "final" values indicate the overall duration of the experiment and the percentage of bleached colonies obtained at the end of the recovery period. The duration of the recovery period is given by the difference between the initial and the final times. At the end of the experiment, the values for the percentage of bleached colonies had been constant, or decreasing very slowly, for the last 24 h.

Expt. No.	*Initial*		*Final*	
	Time h	*% Bleached colonies*	*Time h*	*% Bleached colonies*
1	49	13	119	2
2	70	21	168	2
3	70	28	142	1
4	69	31	138	7
5	70	44	138	10
6	74	70	150	10

TABLE V

RECOVERY AFTER AN ADVANCED STATE OF BLEACHING

2 ml green cells in 200 ml broth at 34.5°. After 138 h culture placed at room temp.

	Bleaching				*Recovering*
Time (h)	70	91	115	138	170
% green colonies	56	20	0	0	5

CONCLUDING REMARKS

The results presented in this report indicate the presence in *Euglena gracilis* of a hereditary system necessary for the formation of chloroplasts. The multiplication of this system can be inhibited by the bleaching temperature, and it can resume when the cells are returned to normal temperature. Streptomycin can cause bleaching of *Euglena* under similar circumstances[4], and it is very likely that the same system is affected. Induction of bleaching by u.v. irradiation has also been reported[7a, b].

The hereditary system exhibits three important characteristics: (a) It possesses an autonomous replication mechanism. (b) It is highly sensitive to environmental factors. (c) It can be lost from partially depleted cells undergoing rapid multiplication*. This loss is apparently due to the rate of multiplication of the system being slower than that of the recovering cells. Whether the normal replication rate of this system is somewhat slower than that of the green cells is not known. The fact that in normal growth curves, the chlorophyll synthesis lags behind that of other cellular constituents (see Fig. 4 in a previous publication[5]) suggests that this might be the case.

The lack of chemical and cytological information about this system prevents a final decision concerning its possible particulate nature. The behavior of the system can, however, be best discussed in terms of the occurrence of a definite number of units or particles in the normal cells. Since this system controls the formation of chloroplasts, its structural relation to the plastids can be examined in the light of our results. Several hypotheses may be considered. (a) Chloroplasts or leucoplasts are self-reproducing organelles, and the particles affected by bleaching are identical with the plastids. (b) The particles are early precursors of the chloroplasts (proplastids). (c) The particles control a step in the formation of chloroplasts, but are located outside the plastids.

In a study of the bleaching of *Euglena* induced by streptomycin, DE DEKEN-GRENSON AND MESSIN[4] concluded that it is the multiplication of the chloroplasts themselves that is affected; but their results are not contradictory to the other possibilities considered here. Our findings, though not incompatible with those presented by these authors, clearly point to the necessity of distinguishing between the self-reproducing system and the chloroplast. When the bleaching cells are returned to room temp., a rapid multiplication of the particles occurs, as demonstrated by the plating experiments. A considerable length of time elapses, however, before the green plastids begin to appear. This distinction between the two effects is most striking when the recovery occurs in the resting medium. As has been shown, the recovery of the hereditary character is enhanced and the formation of chloroplasts strongly repressed under these conditions (see Tables I and III). The lack of relation between the chlorophyll content of the cells and their capacity to form green colonies is illustrated in Table I. These observations rule out the first hypothesis, which identifies the self-reproducing particle with the chloroplast itself; they are compatible with both remaining hypotheses. In either case, multiplication of the particles could take place before the formation of chloroplasts. The previously reported finding, however, that the number of chloroplasts increases linearly with time during the bleaching process[5] makes the third possibility more likely. If the particles were early precursors of chloroplasts (hypothesis (b)), the inhibition during bleaching should result in either a complete block in the formation of chloroplasts or in a slower, but still autocatalytic, rise. In the case of the third hypothesis, a linear increase could be easily explained by assuming that the autonomous system in question controls the catalysis of one of the steps in the development of chloroplasts, as discussed previously[5].

It is possible to attempt an approximate estimate of the number of self-duplicating units present in normal cells. After a sixtyfold increase in the number of cells during the bleaching treatment, the majority of the organisms is not yet irreversibly affected and must still possess at least one hereditary unit. If one assumes that the bleaching temperature completely blocks the multiplication of these units, one can estimate their number in normal cells as no less than sixty. The effect of bleaching could, however, be limited to a retardation of the multiplication of the system, in which case the number of self-reproducing units would be lower.

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* The lack of reversion of this chloroplast deficiency has been demonstrated through 105 weekly transfers in liquid culture[8]. In our laboratory, bleached cells were carried in liquid culture for 2 ½ years without the appearance of any green cells. This complete lack of reversion indicates that the deficiency is the result of the loss rather than the inactivation of the factor responsible for this hereditary character.

In conclusion, a few remarks of more general import should perhaps be added. Starting from the evidence for the existence in *Euglena gracilis* of a system endowed with autonomous self-duplication, one may ask to what extent such systems participate in the general processes of heredity. On the basis of our present knowledge, the *Euglena* system could best be described as a hereditary factor or particle, presumably nonchromosomal, characterized by possessing a replication mechanism autonomous in respect to that of the cell as a whole and by exhibiting a very marked susceptibility to environmental conditions which leads to irreversible changes in the properties of the cell. One analogous case can be cited, *viz.*, the cytoplasmic factor that controls the formation of respiratory enzymes in yeast[9]. This factor is affected by acriflavine[9], elevated temperature[10], u.v. irradiation[11,1] and caffeine[13]. The recognition of its loss, moreover, is also conditional on the multiplication of the cells. No other examples of such a system appear to have been recorded; but it is not unlikely that additional instances of similar self-duplicating systems will be recognized, especially when some of the effects of streptomycin and u.v. irradiation are reexamined in the light of our present findings.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the very able technical assistance of Miss C. A. REBMAN.

This work was supported by research grants from the American Cancer Society and the National Science Foundation.

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PARTITION OF VIRUS PARTICLES IN A LIQUID TWO-PHASE SYSTEM

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(Received April 7th, 1959)

SUMMARY

1. The partition coefficients of *Helix pomatia* hemocyanin, tobacco mosaic virus, the bacteriophages T_3, T_2 and T_4, and vaccinia virus have been determined in the phase system dextran–methylcellulose–water.

2. A proportionality between the logarithm of the partition coefficient and the surface area of the particles was found, in accordance with the Brønsted theory on the partition of macromolecules in two-phase systems.

3. The possibility of using polymer phase systems for the analysis and fractionation of virus suspensions is shown by a counter-current experiment with tobacco mosaic virus.

INTRODUCTION

In a previous publication[1] the partition of some proteins in a liquid two-phase system, composed of dextran, methylcellulose and water, was described. A correlation between the size of the protein molecules and their partition coefficients was found (partition coefficient, K = concentration in the top phase divided by the concentration in the bottom phase).

Both from a theoretical and a practical point of view it is of great interest to study the relation between the partition coefficient and the size of rather large molecules and particles. We have therefore determined the partition coefficient for three bacteriophages T_2, T_3 and T_4. For this problem the great advantage of these particles lies in the possibility of determining small amounts of them relatively accurately by a specific method with the agar layer technique in which each phage gives rise to a countable plaque. It is thus possible to determine even very small partition coefficients where the concentration in the top phase is very low. Since the phase system used was not exactly the same as in previous experiments, the partition coefficients of *Helix pomatia* hemocyanin and tobacco mosaic virus (TMV) have been redetermined in the same system as the bacteriophages. In addition the partition coefficient of vaccinia virus has been determined.

EXPERIMENTAL

Materials

Hemocyanin was obtained from *Helix pomatia* snails collected in the autumn.

The hemocyanin was purified by 3–4 centrifugations in the Spinco L preparative ultracentrifuge (rotor no 40) at 40,000 rev./min for 2 h. The pellet was each time resuspended in water. The repeated centrifugations were necessary in order to remove cellulase activity usually found in the collected blood and which degrades methylcellulose, thus destroying the phase system. In the ultracentrifuge (Spinco analytical model E) 90–95 % of the material gave rise to one peak, while the rest, probably aggregates, gave rise to a faster moving peak.

TMV was obtained in the usual way by differential centrifugation[2]. (We are grateful to Dr. C. A. KNIGHT, Virus Laboratory, University of California, Berkeley, Calif., for a sample of their TMV preparation.) Before use the TMV was centrifuged once in 0.01 M phosphate buffer pH 7.5, containing 0.01 M EDTA[3] twice in 0.01 M phosphate, pH 7.5, at 30,000 rev./min for 45 min (in rotor no 30 of the Spinco model L centrifuge) and then once at 10,000 rev./min for 15 min. In the ultracentrifuge 70 % of the material representing the monomer unit was estimated to give rise to a main peak and 10 % to a faster peak which is formed by aggregates. 20 % of the material was considered to be split products.

The phage suspensions used were filtered lysates after growth of T_2, T_3 and T_4 on *Escherichia coli*, strain B. Purified preparations of T_3 and T_4 were also used without any observed difference in the results. (We are indebted to Dr. O. MAALØE, Statens Seruminstitut, Copenhagen, for the original phage stocks.) T_2 and T_3 were grown in Friedlein buffer, T_4 in Friedlein buffer plus tryptophan.

Dextran was obtained from AB Pharmacia, Uppsala, Sweden. Intrinsic viscosity 0.68 dl/g.

Methylcellulose, "Methocel, 31.5 % methoxyl, visc. 4149 cps." was obtained from Dow Chemical Company, U.S.A.

The phase systems

A phase diagram of the dextran–methylcellulose–water system has been described elsewhere[1]. Our experiments have been carried out in two systems, here denoted as A1 and A2. They are both near the plait point of the phase diagram and similar to system A previously described[1]. The only difference between the two systems is the volume ratio of the two phases. Data on system A1 are given in Table I. (The method used for the determination of the composition of the two phases will be described elsewhere.) System A2 is obtained by mixing 20 g of the bottom phase

TABLE I

THE COMPOSITION OF THE PHASES IN THE SYSTEM DEXTRAN–METHYLCELLULOSE–WATER

Total composition: 0.68 % (w/w) dextran; 0.36 % (w/w) methylcellulose; 98.96 % (w/w) water.

Temperature	*Phase*	*Dextran* % *w/w*	*Methylcellulose* % *w/w*	H_2O % *w/w*
+ 20°	Bottom phase	1.18	0.18	98.64
	Top phase	0.47	0.43	99.10
+ 4°	Bottom phase	1.33	0.18	98.49
	Top phase	0.51	0.43	99.06

The volume ratio top phase/bottom phase is 2.9 at + 20° and 3.8 at + 4°.

from system A1 with 5 g of system A1, both being at the same temperature. The composition of the two phases will therefore be identical for the two systems.

Partition experiments

In the partition experiments H_2O was replaced by an equal weight of 0.15 *M* NaCl and 0.01 *M* buffer, except in the experiments with TMV when NaCl was omitted. Sodium phosphate was used as buffer in all experiments except with hemocyanin at pH 8.2 when tris(hydroxymethyl)aminomethane–HCl was used. Because of the high viscosity, the polymer phase systems were prepared by weighing in the polymer solutions. This was conveniently done on an automatic balance (Mettler type K7).

A partition experiment with system A1 was carried out in the following way: 36 g of a 1% (w/w) solution of methylcellulose, 6.8 g of a 10% (w/w) solution of dextran, 15 g 1 *M* NaCl, 10 g 0.1 *M* buffer and 12.2 g H_2O (total = 80 g) were weighed in a flask. This mixture is kept in the cold as a stock mixture. Under sterile conditions the mixture can be kept for several weeks. For use 4 parts are diluted with 1 part of virus suspension to give the desired phase system. After the flask had been gently shaken in order to mix the two phases a sample was immediately taken out with a pipette with a wide tip. 4 g of the stock mixture were weighed in a tube 7 cm long and 1 cm in diameter. 1 g of the phage or protein suspension was then added to the tube. The tube was equilibrated in a thermostat at +20.0° or in a cold room at +4°, subsequently inverted about 50 times and then allowed to stand for 30 h at +20° or for 48 h at +4° for phase separation. A sample of about 0.5–1 ml of the top phase was taken out with a pipette and an equal amount of the bottom phase was obtained by carefully dipping a narrow pipette through the top phase down into the lower part of the bottom phase, waiting a few minutes and then slowly sucking up a sample.

An experiment on system A2 was done in the following way. First 800 g of the stock mixture was diluted with 200 g H_2O, mixed and allowed to stand for phase separation. The bottom phase was then collected. 20 g of this was weighed in a tube 13 cm long and 1.7 cm in diameter. 4 g of the stock mixture and 1 g of phage suspension were then added. The experiment was then continued as with system A1.

The concentration of phages was determined by the usual agar layer technique[4]. To obtain good reproducibility the dilution of the phage suspensions to be measured was carried out so that the number of plaques was 200–300 for T_2 and T_4 and between 50–80 for T_3. All dilutions were made in Friedlein buffer alone, except for T_4 where tryptophan was added to the buffer. The titer determinations were started immediately after the collection of the samples from the two phases. The determinations were repeated 24 h later, the phage samples being kept in the cold in the interval. No significant differences were found between the determinations, showing that virus inactivation, if any, occurred at similar rates in the top and the bottom phases. If, however, the original separation of the two polymers was allowed to take place at +20° a correction was necessary to adjust for a difference in inactivation rates in the top and the bottom phases. This correction was determined by following the infectivity in each polymer system separately for 3 days by one titer determination each day.

The correction found was, for a time of separation of 36 h at 20°: for T_2 — 12% of *K* (pH 5, 6.2); the corrections for T_4 and T_3 were of the same order of magnitude.

In the experiments the phage titers were varied between 10^7–10^{12} in parallel experiments. The stock solutions showed good stability in the cold for several weeks.

In the experiments with hemocyanin and TMV, protein was determined by the method of LOWRY *et al.*[5]. The samples were, if possible, chosen so that equal amounts of protein were analysed from both the bottom and top phases in order to eliminate error due to the non-linearity of extinction versus protein concentration. Some methylcellulose precipitated during the analysis and was removed by filtration. In addition some of the analyses were checked by nitrogen determinations with the Kjeldahl method.

Counter-current distribution experiment (TMV)

Since the TMV preparation is polydisperse the *K*-value determined on the whole TMV preparation will be an average value of the *K*-values of all the different molecular species present in the preparation. In order to determine the *K*-value for the monomer unit (300 × 15 mμ) a counter-current distribution[6] was carried out in the following way. 6 mg TMV was first distributed in 20 g of system A1 with 0.01 *M* sodium phosphate pH 7.1. The volumes of the bottom phase and the top phase were 5 and 15 ml respectively. After phase separation the top phase was removed and added to a second tube with 5 g of the bottom phase. Fresh top phase was then added to the first tube. The tubes were shaken and after phase separation the top phase of the second tube was added to a third tube with 5 g of the bottom phase. The top phase of the first tube was moved to the second tube and fresh top phase added to the first tube. The same operation was then repeated with the addition of a fourth tube.

The protein content was then determined in each phase of all the tubes. It was found that 82 % of the total material was left in the first tube with a *K*-value of 0.014. In the second tube 15 % of the total was present and had a *K*-value of 0.044. In the third and fourth tubes 2 and 1 % respectively of the material was found and this amount was too small for accurate determination of the *K*-value. We now consider that the 15 % of the material which is present in the second tube consists in a large part of the split products of TMV while mainly the monomers and aggregates remain in the first tube. We thus have a *K*-value of 0.014 for the mixture of monomers and its aggregates. The aggregates are considered to be 15 % of this mixture and to have a surface area of at least twice that of the monomer. Hence they should have about the same *K*-value as T_2 in the same system, *i.e.* 0.0026. The concentration of the aggregates in the two phases can thus be calculated and substracted from the determined concentration, when the *K*-value for the monomer is found to be 0.016. This value must be regarded as somewhat uncertain, but we consider it highly probable that the true *K*-value for the monomer unit lies somewhere between 0.01 and 0.02, as is shown in Fig. 2.

RESULTS AND DISCUSSION

It is a generally observed phenomenon that large-molecular-weight substances yield a one-sided distribution in a two-phase system. This has been studied by BRØNSTED, both theoretically[7] and also experimentally by BRØNSTED AND WARMING[8] with inorganic colloids in low-molecular-weight phase systems. Experiments with proteins, also in low-molecular-weight phase systems, demonstrated that proteins usually collected in one of the phases. (See the reviews by PORTER[9] and VON TAVEL AND SIGNER[10] for further references.) It was possible to obtain measurable partition coefficients only by working near the plait point of a phase system, when the two phases become very similar. In polymer–polymer phase systems, however, the tendency for proteins to give one-sided distributions is not so pronounced as in low-molecular-weight phase systems. Thus experiments with proteins in the system dextran–methylcellulose–water[1] showed that well-defined partition coefficients around unity could easily be obtained even at compositions of the phase system which were not near the plait point.

In this investigation we have studied extremely large molecules, virus particles. Theoretically their tendency to give a one-sided distribution should be still more pronounced than that of ordinary proteins. In order to obtain measurable partition coefficients of the virus particles it has been necessary, therefore, both to use a polymer–polymer phase system and to work near its plait point at the same time.

The results from the measurements are found in Tables II and III. All particles have K-values below unity, that is they partition in favour of the dextran-rich bottom phase. The larger the size of the particles, the smaller the K-values. These results are essentially similar to those found for proteins in the same phase system[1], and

TABLE II

PARTITION COEFFICIENTS, K, OF HEMOCYANIN AND SOME VIRUS PARTICLES IN THE SYSTEM DEXTRAN–METHYLCELLULOSE, SYSTEM A1, + 20°

Particle	*pH*	*K*	*–log K*
Hemocyanin "eighth"	8.2	0.63	0.20
Hemocyanin whole	5.9	0.25	0.60
TMV	7.1	$2.5 \cdot 10^{-2}$	1.60
T_3	6.4	$3.2 \cdot 10^{-2}$	1.50
T_2	4.6	$2.9 \cdot 10^{-3}$	2.54
	5.3	$2.5 \cdot 10^{-3}$	2.60
	6.2	$2.6 \cdot 10^{-3}$	2.59
	6.4	$2.6 \cdot 10^{-3}$	2.59
	7.4	$2.8 \cdot 10^{-3}$	2.55
T_4	6.5	$2.9 \cdot 10^{-3}$	2.54

TABLE III

PARTITION COEFFICIENTS, K, OF HEMOCYANIN AND SOME VIRUS PARTICLES IN THE PHASE SYSTEM DEXTRAN–METHYLCELLULOSE AT + 4°

Particle	*System*	*pH*	*K*	*–log K*
Hemocyanin whole	A1	5.8	0.25	0.60
TMV	A1	7.1	$2.6 \cdot 10^{-2}$	1.59
T_3	A1	6.6	$2.1 \cdot 10^{-2}$	1.68
T_3	A2	6.6	$2.3 \cdot 10^{-2}$	1.64
T_2	A1	7.3	$2.6 \cdot 10^{-3}$	2.59
T_2	A2	5.7	$6–10 \cdot 10^{-4}$	3.0–3.2
T_2	A2	6.9	$4–6 \cdot 10^{-4}$	3.2–3.4
T_4	A1	6.9	$2.5 \cdot 10^{-3}$	2.60
T_4	A2	6.9	$3–5 \cdot 10^{-4}$	3.3–3.5
Vaccinia	A1	6.8		2.8 ± 0.3
Vaccinia	A2	7.0		4.1 ± 0.3

have been analysed in terms of BRØNSTED's theory. BRØNSTED deduced the following formula: $\log K = \lambda M/RT$ where K = partition coefficient, = R the gas constant, T = temperature and λ is a constant for a given phase system and for isochemical substances, *i.e.* substances which differ in molecular or particle weight only. When the partitioned substance is globular M should be replaced by the surface area of the molecules. There was good agreement with theory for proteins in similar systems to that used here, the logarithm of the K-value being approximately proportional to $M^{2/3}$, which in turn is approximately proportional to the surface area of the molecules[1]. Since the shape and dimensions of the particles studied here are known from measurements with the electron microscope we have attempted to calculate the surface area of the particles and to relate this to the K-values. The calculation of the surface area was carried out as follows.

For hemocyanin the data of SCHRAMM AND BERGER[11] were used. The two types of hemocyanin molecules at pH 4.7–7.0 and 8.2–9.3 were assumed to have a form of a rectangular parallelepiped, with the sides 40 × 20 × 10 mμ for the whole molecule (pH 4.7–7.0) and 20 × 10 × 5 mμ for the "eighth" molecule (pH 8.2–9.3).

TMV was assumed to be a cylinder 300 mμ long and 15 mμ in diameter[3].

The form and dimensions for T_2, T_3 and T_4 have been studied by WILLIAMS AND FRASER[12] who concluded that the heads of all three phages may have the form of either a rhombic dodecahedron or a hexagonal prism with bipyramidal ends. We have chosen the latter alternative. The tails have been regarded as cylinders. For T_3 the distance between parallel sides is 47 mμ. All the sides of both the prism and the pyramids are of an equal length $(47/\sqrt{3})$. The cylinder is 15 mμ long and 10 mμ in diameter. For T_2 and T_4 the maximal length of the head is 95 mμ and the maximal width 65 mμ. The ratio between the length of the prism and the side of the pyramids is 3:2. The tail is 100 mμ long and 25 mμ in diameter.

The results from the calculation may be found in Table IV. Log K has been plotted against the calculated surface areas in Figs. 1 and 2. In Fig. 1 (system A1, +20°) it may be seen that log K is proportional to the surface area of the particles up to the size of T_3. The same relation holds for system A1 at +4° (see Fig. 2). For larger particles, however, the curve deviates from a straight line and a limiting value is obtained about the size of T_2. This has also been found for other virus particles of similar size, now being studied. We consider that this plateau in the curve is due to small drops of the bottom phase present in the top phase. It is to be noted that

TABLE IV

SURFACE AREA OF DIFFERENT PARTICLES CALCULATED FROM ELECTRON MICROSCOPE DATA

Particle	*Surface area* $(m\mu)^2 \times 10^{-3}$
Hemocyanin (*Helix pomatia*)	
"eighth" molecule	0.86
whole molecule	3.5
TMV	14.4
T_3	8.7
T_2	25.5
T_4	25.5
Vaccinia	220

small drops of the bottom phase may remain in the top phase for a long time due to the small density difference between the two phases and the comparatively high viscosity of the top phase which reduces their sedimentation rate. These drops of the bottom phase which are rich in virus particles will give a more or less constant contribution to the number of virus particles present in the top phase. This contribution will be more important the lower the K-values since the number of the virus particles present in the ideal top phase will then be smaller compared with the number of the virus particles in the drops. This effect of the drops is negligible in the case of hemocyanin, T_3 and TMV but it probably entirely predominates over the real K-values of T_2, T_4 and Vaccinia. In order reduce this source of error we chose the system A2, which has exactly the same composition of the two phases as A1 but with a different volume ratio. The volume of the bottom phase of system A2 is thus about six times larger than the top phase, while in system A1 the bottom phase is only about one third of the top phase. It has been observed in all aqueous polymer–polymer phase systems studied so far[13] that the amount of emulsion depends very much upon the volume ratio. It is also a general phenomenon that if one of the phases is small compared with the other, then the smaller phase is much clearer optically, while the larger phase contains small drops of the other. It may be expected, therefore, that the top phase of system A2 should contain a smaller amount of drops of the bottom phase. As may be seen from Table III and Fig. 2 the observed K-values of T_2, T_4 and Vaccinia but not that of T_3 have been considerably reduced in system A2. The linear relation between log K and the surface area exists up to the size of T_2 and T_4 in system A2. In conclusion, therefore, the behaviour of hemocyanin, T_3, T_2, T_4, and TMV in the dextran-methylcellulose system, is in good agreement with the Brønsted formula and the surface area of the particles mainly determines the partition coefficient.

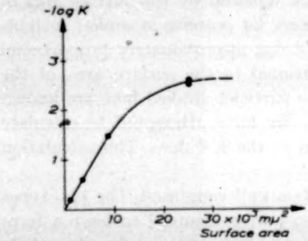

Fig. 1. The relation between the partition coefficient, K, and the surface area of the partitioned particles in the liquid two-phase system dextran–methylcellulose–water (system A1). The points represent from the left: hemocyanin "eighth", hemocyanin whole, T_3, T_2–T_4. Temperature + 20°. Compare Table II and IV.

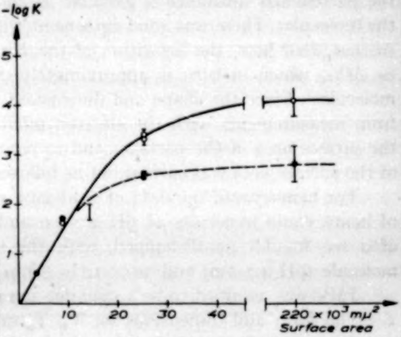

Fig. 2. The relation between the partition coefficient, K, and the surface area of the partitioned particles in the liquid two-phase system dextran–methylcellulose–water. Solid circles in system A1, open circles in system A2. The points represent from the left: hemocyanin whole, T_3, T_2–T_4 and Vaccinia. The value for TMV by the counter-current experiment is indicated by a vertical line. Temperature + 4°. Compare Table III and IV.

The T_2 titers of the top phase were not so reproducible in A2 as in A1. This is not surprising if the relatively small numbers (20,000) of phage particles per ml are considered. Adsorption at interfaces—liquid/air interface, glass walls of the tube and pipettes for example—will then have more influence on the concentration.

The *K*-value is not much affected by changes in the temperature, which is of great practical advantage. Because of the low *K*-values of the viruses this phase system can be used for enrichment of diluted virus suspensions, as has been described elsewhere[14]. The same phase system has also been used for fractionation of microsomes[15].

No significant difference in the *K*-value for T_2 at different pH were found. The form of the curve at T_2 makes any conclusions uncertain. The experiments in system A2, however, do not favour a great increase of particle surface such as would take place if T_2 aggregated to dimers at lower pH values[16,17]. (To correlate these experiments with published ultracentrifugation data, it may be necessary to investigate the influence of the components of the phases.)

The counter-current experiment with TMV described here indicates that polymer-polymer phase systems may be used for the analysis of mixtures of rather large particle weight substances. This possibility is now being studied further.

ACKNOWLEDGEMENTS

We wish to thank Professor A. Tiselius for his kind interest in this work and Dr. Philipson, Department of Virology, the Institute of Hygiene and Bacteriology, Uppsala, for the supply and titration of Vaccinia virus.

This work has been supported financially by grants from the Technical and Natural Science Research Councils of Sweden and the U.S. Department of Army, through its European Office, under contract number DA-91-508-EUC-287.

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EVIDENCE OF THE OCCURRENCE OF A LARGE RNA MOLECULE PER RIBONUCLEOPROTEIN PARTICLE

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(Received April 4th, 1959)

SUMMARY

A purified RNA preparation consisting of two sedimenting components has been obtained from mouse brains. The molecular weights of these two components, as determined from the sedimentation and viscosity data, are $1.7 \cdot 10^6$ and $0.7 \cdot 10^6$, respectively. The larger value is equal to the RNA content of one mole of the larger tissue ribonucleoprotein. This result supports the previously advanced hypothesis that one RNA particle with a molecular weight of about $1.7 \cdot 10^6$ is contained in one molecule of a certain class of ribonucleoprotein particle.

INTRODUCTION

An impressive body of evidence has been accumulated to show that viral RNA plays an important role in carrying the genetic information of many viruses[1–6]. In addition, it is generally believed that RNA is involved in protein synthesis, and the site of the synthesis in cytoplasm has been inferred to be ribonucleoprotein (RNP)* particles[7,8]. Accordingly, it has been considered of interest to investigate the state and size of the RNA moiety of the RNP particles.

In a communication two years ago, the author advanced the hypothesis that one RNA particle with a molecular weight of about $1.7 \cdot 10^6$ is contained in one RNP particle of many viruses and a class of cytoplasmic particles in plants and animals[9]. This hypothesis was based on two experimental findings: (a) In the plant and animal viruses so far studied, the average weight of the total RNA is approximately $2 \cdot 10^6$ times the weight of a hydrogen atom per particle of virus[10]. Similar calculations[9] from data available for a class of large cellular RNP particles, 80-S particles, from yeast, pea seedlings and rat liver give a figure of $1.7 \cdot 10^6$**. (b) Determination of the molecular weight of the RNA from tobacco mosaic virus (TMV) consistently gave a value of $1.7 \cdot 10^6$ under a variety of preparative conditions[12]***.

The second experimental finding, at the time the hypothesis was suggested, thus consists of the study of only one system. Since TMV, furthermore, is unusual in being rod-shaped, rather than roughly spherical in shape, it seemed imperative to investigate the existence of RNA with such a molecular weight in other systems. In the present communication the results of such an investigation on mouse brain are reported.

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* The term ribonucleoprotein is used throughout the text to denote also particles containing substances in addition to RNA and protein.

** This figure has recently been determined for 70-S particles from *E. coli* also[11].

*** Electron microscopic evidence has recently been presented to support this hypothesis[13].

MATERIALS AND METHODS

Preparation of RNA

The RNA was prepared at 3° from the brains of 4-day-old Swiss mice according to a modification of the GIERER-SCHRAMM procedure[3,14]. 9 g of the frozen brains were homogenized in an ice-water bath with 20 ml of water-saturated phenol* and 20 ml of 5 g/l sodium citrate. The homogenate was then centrifuged to separate the aqueous solution from phenol and resulting solid materials. The aqueous solution was further extracted twice with an equal volume of phenol by vigorous agitation for 10 min. The final, clear aqueous layer was mixed with two volumes of absolute alcohol for 30 min and was then centrifuged at 3,000 × g for 15 min. The sediment was washed three times each with 30 ml of a solution consisting of alcohol and 5 g/l sodium citrate at a volume ratio of 2 to 1. The washed sediment was then resuspended in a volume, between 9 and 20 ml, of 5 g/l sodium citrate. Such a preparation was found to contain DNA at a concentration about 15% that of RNA as estimated by diphenyl amine reaction[15]; the DNA present was of high molecular weight with an average intrinsic viscosity of about 60 dl/g. The RNA was further precipitated three times from this preparation by being maintained with 1.0 M NaCl for 30 min

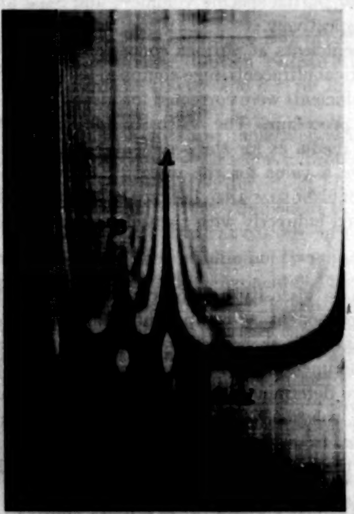

Fig. 1. Sedimentation diagram of a purified RNA preparation from mouse brain. Total RNA concentration was 0.17%. Medium was 0.5% sodium citrate. A and B are the fast- and slow-sedimenting components, respectively.

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* The phenol had been washed four times with an equal volume of demineralized water before use.

The final precipitate was resuspended in citrate solution. Such a purified RNA preparation contained less than 10 μg of DNA in 3 mg of RNA. As is seen from the schlieren pattern in Fig. 1, it contained two sedimenting components*.

Isolation of the slow-sedimenting component

The slow component, B, was isolated from the RNA preparation in order to facilitate the characterization of the two components. The isolation was performed at 8° in a separation cell according to the YPHANTIS-WAUGH procedure for characterization by direct measurement of activity[16], except that the RNA preparation was centrifuged under such conditions that at the end of centrifugation the solution above the partition contained practically all of the slow component of the preparation and nothing else. The solution above the partition was then carefully taken out of the cell.

Determination of molecular weights

It has been inferred that the RNA prepared from the RNP particles of calf liver behaves like a randomly coiled single-chain molecule[17]. Hence, the molecular weight, M, of each RNA component was calculated from sedimentation constant, $S_{20,w}$ and intrinsic viscosity, $[\eta]$, according to the equation of MANDELKERN AND FLORY for a flexible chain molecule[18]. The equation can be reduced into the following form

$$M = 1.34 \cdot 10^4 \times S_{20,w}^{1.5}[\eta]^{0.5} \tag{1}$$

when 0.578 is taken for partial specific volume[19], and $S_{20,w}$ and $[\eta]$ are expressed in Svedbergs and dl/g respectively.

Sedimentation coefficients at various concentrations were measured at 5° in a Spinco Model E analytical ultracentrifuge equipped with a thermal regulating system. The sedimentation coefficients were corrected for viscosity and temperature according to the conventional procedure. The viscosity measurements were performed at $3 \pm 0.1°$ in the same media as for the sedimentation studies, with a 1-ml Ostwald viscometer which gave a value for the viscosity of 20% sucrose solution in good agreement with that of BINGHAM AND JACKSON[20]. The viscosity of the component A solution was determined indirectly with the following equation:

$$\eta_1 c_1 - \eta_0 = (\eta_3 c_3 - \eta_0) - (\eta_2 c_2 - \eta_0) \tag{2}$$

where η and c designate viscosity and concentration, and the subscripts 0, 1, 2, 3 denote the solvent, component A, component B and the whole RNA preparation, respectively. This equation is theoretically expected to hold at dilute solutions and has been verified experimentally with some systems of proteins[21, 22]. The concentrations c_2 and c_3 were determined by the O.D. at 258 mμ, using an extinction coefficient of 225 for 1% solution[23]. The concentration c_1 was then determined by subtracting the value of c_2 from that of c_3. The value of c_1/c_2 so obtained, 2.4, was in good agreement with the ratio of the areas associated with the two components on a schlieren diagram**. The $S_{20,w}$ and intrinsic viscosity were determined by extrapolating the measured values $1/S$ and $[\eta]$ to infinite dilution.

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[*] It should be pointed out that the purification procedure used removed all RNA of low molecular weight from the preparation.

[**] The area of component B had been corrected for the JOHNSTON-OGSTON effect[24, 25].

TABLE I

COMPONENT A OF RNA

c_1/g/dl	$(S_{20,w})_1$ Svedberg	$[\eta]_1$ dl/g
0.14	25	0.84
0.07	28	0.74
0.04	28.5	0.69
Extrapolated to infinite dilution	30	0.61

TABLE II

COMPONENT B OF RNA

c_2/g/dl	$(S_{20,w})_2$ Svedberg	$[\eta]_2$ dl/g
0.05	19.5	0.42
0.03	19.0	0.40
Extrapolated to infinite dilution	19.5	0.41

RESULTS AND DISCUSSION

Tables I and II present the data from which $S_{20,w}$ and $[\eta]$ of the two components were obtained by extrapolation to infinite dilution. Substitution of these values in eqn. 1 resulted in molecular weights of $1.7 \cdot 10^6$ and $0.7 \cdot 10^6$ for components A and B respectively.

Since the RNA from TMV had a constant molecular weight, whether isolated by the phenol method or by other procedures[26], it seems improbable that component A was an aggregation product of the isolation procedure used. Two recent findings might be considered as favoring the possibility that component A was an aggregate of component B through the effect of some divalent cations such as Mg^{++}. First, Mg^{++} has been reported to reversibly effect the formation of two- and three-stranded polyribonucleotides from their single-stranded components[27]. Second, ultracentrifugal studies on the 70S–80S RNP particles from yeast[28], bacterium[11] and pea seedlings[29] have been interpreted as showing that these particles can be reversibly decomposed into a few RNP subunits by a lowering of the Mg^{++} concentration of the medium. The possibility that such mechanisms were an important factor in the present experiments is considered to be unlikely, however, because the ratio of concentrations of the two components was not significantly changed when (a) the RNA preparation was precipitously aggregated once by being maintained at pH 1.64 for 10 min at 3°, (b) 1 *M* NaCl precipitation was used once as well as 4 times in the procedure for the RNA preparation, or (c) 0.01 *M* phosphate, pH 7.0, was used instead of sodium citrate as a medium for RNA. If the Mg^{++} were reversibly bound, one would expect a change in the amount of Mg^{++} on an RNA particle in each of these cases. It is concluded, therefore, that RNA with a mol. wt. of $1.7 \cdot 10^6$ exists *in vivo* and in abundant amount.

The very recent work of Gierer[30], which came to the author's attention during the preparation of this manuscript, has demonstrated two RNA components with molecular weights of $1.8 \cdot 10^6$ and $0.6 \cdot 10^6$, respectively, in RNA preparations from tobacco leaves and from the microsome fraction of rat liver. Thus, RNA with a mol. wt. of about $1.7 \cdot 10^6$ has been shown to be present in two tissues from animal and plant[*] as well as in one kind of spherical animal cytoplasmic RNP and a rod-shaped plant virus, TMV. These results strengthen the hypothesis.

It has not been possible in these experiments to determine the origin of the RNA particles of smaller molecular weight, component B. It seems equally possible that they arise from RNP particles of small size known to be present in cytoplasm, or that they represent degradation products of the 80-S particles.

ACKNOWLEDGEMENTS

The author is indebted to Drs. H. G. Kunkel, L. C. Craig and D. A. Yphantis for making their ultracentrifuges available. The technical assistance of Miss H. J. Scherer is also acknowledged.

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[*] An average molecular weight of about 1 million has been reported for a high molecular weight fraction of the RNA from Ehrlich ascites tumor cells[31]. Since the fraction was obtained by precipitation once with 1 *M* NaCl, it undoubtedly contained both the fast and the slow components. Therefore, the reported value of about 1 million is an average value for the molecular weight of a mixture of the fast and the slow components.

STUDIES ON THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID BY EXTRACTS OF MAMMALIAN CELLS

III. NET SYNTHESIS OF POLYNUCLEOTIDES

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(Received April 6th, 1959)

SUMMARY

By degradation experiments with appropriate enzymes it has been shown that the incorporation of labelled precursors into DNA by cell-free extracts of Ehrlich ascites tumour cells does not represent mere terminal addition of single or multiple nucleotide units to an existing DNA chain but represents true synthesis of new polynucleotide.

Net synthesis of polynucleotide amounting to more than 70 % of the amount of carrier DNA present has been demonstrated by incubation of the deoxynucleoside-5′-monophosphates of adenine, guanine, cytosine and thymine with ATP and the ascites cell extract. When the corresponding triphosphates are used, ATP may be omitted.

INTRODUCTION

In previous papers[1, 2] we have described the incorporation of [^{3}H]TDR into DNA by soluble extracts of Ehrlich ascites tumour cells. The present paper deals with experiments designed to determine the intramolecular location of the [^{3}H]TDR and of [^{32}P]dCMP and TMP incorporated into DNA in this type of experiment. The net synthesis of DNA by soluble extracts of Ehrlich ascites tumour cells is also described.

METHODS

Enzymes

Crystalline DNase I from pancreas was purchased from Nutritional Biochemicals Inc.

DNase II was prepared from calf spleen as described by MACDONALD[3]. The product obtained was found to have 3′-phosphodiesterase and phosphomonoesterase

The following abbreviations are used: DNA for deoxyribonucleic acid, RNA for ribonucleic acid, dAMP, dGMP, dCMP and TMP for the 5′-monophosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine respectively, dATP, dGTP, dCTP and TTP for the 5′-triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine respectively, TDR for thymidine, ATP for adenosine triphosphate, DPN for diphosphopyridine nucleotide, DNase for deoxyribonuclease, and tris for 2-amino-2-hydroxymethyl propane-1, 3-diol.

activities in addition to that of DNase II. It was therefore chromatographed on Celite 545 according to KOERNER AND SINSHEIMER[4] to remove the phosphomonoesterase. The final product contained both DNase II and 3′-phosphodiesterase contaminated with a small amount of phosphomonoesterase. When it was allowed to act on DNA, the products of digestion behaved on a Dowex-1 formate column as deoxyribonucleoside monophosphates and were hydrolysed by 3′-nucleotidase from rye grass (generously supplied by Dr. L. HEPPEL) to deoxyribonucleosides and inorganic phosphate.

The purification of 5′-phosphodiesterase from Russell viper venom was carried out according to the method of COHN, VOLKIN AND KHYM[5] by Mr. R. BURDON.

Preparation of ascites DNA

Ascites DNA was prepared by the method of KAY, SIMMONS AND DOUNCE[6].

Preparation of labelled DNA

[^{3}H]DNA was prepared as previously described[2] by incubating a number of flasks containing 20 mg soluble Ehrlich ascites cell protein, 30 µmoles ATP, 3.0 µC [^{3}H]-TDR, 750 µg DNA, 1.5 µmoles DPN, 30 µmoles $MgCl_2$ and 600 µmoles tris buffer pH 7.9 in a total vol. of 6 ml. Incubation was carried out in air at 37° for 2 h with shaking.

[^{32}P]DNA was prepared by incubating a number of flasks containing 7 ml whole ascites tumour cell suspension, 80 µmoles glucose and 350 µC [^{32}P]orthophosphate. Incubation was continued in air for 6 h at 37° with shaking.

DNA containing either [^{32}P]dCMP or [^{32}P]TMP was prepared by incubating a series of flasks containing 20 mg soluble Ehrlich ascites cell protein, 30 µmoles ATP, 750 µg DNA, 1.5 µmoles DPN, 30 µmoles $MgCl_2$, 600 µmoles tris buffer pH 7.9 and 0.5 µmoles of either [^{32}P]dCMP or [^{32}P]TMP containing approx. 30,000 counts/min. Incubation was for 2 h in air at 37° with shaking.

At the end of the incubations, the labelled DNAs in all cases were isolated by the procedure previously described[2].

Degradation of DNA to nucleoside-5′-monophosphates and to nucleoside-3′-monophosphates

[^{32}P]dCMP and [^{32}P]TMP were obtained from [^{32}P]DNA (as nucleoside 5′-phosphates) after digestion with DNase I and 5′-diesterase as described by COHN, VOLKIN AND KHYM[5]. The digest was treated with $HClO_4$ to give a final concn. of 0.5 N and the precipitated material was removed by centrifugation. The clear supernatant fluid was neutralised with 5 N KOH and after removal of the insoluble $KClO_4$ was used for separation of the constituent nucleotides by ion exchange chromatography as described below.

For the degradation of DNA to deoxyribonucleoside 3′-monophosphates, approx. 7.5 mg of DNA was incubated at 37° for 18 h in 2 ml 0.33 M formic acid–ammonium formate buffer pH 4.5 along with 1 ml of the DNase II, 3′-diesterase preparation. The reaction was stopped by the addition of cold $HClO_4$ to a final concn. of 0.5 N, acid insoluble material was removed by centrifugation and the extract was neutralised with 5 N KOH. The precipitated $KClO_4$ was removed and the soluble material was chromatographed on Dowex-1 formate columns as described below.

Some samples of DNA were digested directly with 5′-phosphodiesterase under the conditions described by ADLER, LEHMAN, BESSMAN, SIMMS AND KORNBERG[7]. At various time intervals samples of the reaction mixture were removed, treated with $HClO_4$ to a final concn. of 0.5 *N*, the insoluble material centrifuged down and washed twice with 0.5 *N* $HClO_4$. The clear supernatant fluid was neutralised with 5 *N* KOH. The insoluble residue of DNA and protein was extracted 3 times with 0.5 *N* $HClO_4$ at 70° for 20 min and the extracts pooled. The extracts were neutralised with 5 *N* KOH and aliquots were taken for measurement of radioactivity and u.v. absorption at 268 mμ.

Isolation of deoxyribonucleotides

The acid-soluble material obtained on the digestion of DNA with DNase I and 5′-phosphodiesterase or DNase II and 3′-phosphodiesterase was applied to Dowex-1 formate columns 6 cm × 1 cm. The columns were washed with water to remove any deoxyribonucleosides and were then eluted successively with 0.05 *N*, 0.3 *N*, 1.8 *N* and 2.5 *N* formic acid to remove dCMP, dAMP, dGMP and TMP respectively. After identification of the nucleotides by their u.v. absorption spectra, the eluates were freeze-dried or freeze-concentrated according to the requirements of the experiment and samples were taken for the measurement of u.v. absorption and radioactivity.

Preparation of deoxyribonucleoside triphosphates

Samples of dATP, dGTP and dCTP were prepared as described by KEIR AND SMELLIE[8]. TTP was prepared chemically by the method of SMITH AND KHORANA[9] and a further sample of dCTP was purchased from the Sigma Chemical Company.

Analytical procedures

The estimation of DNA was carried out as described previously[2]. The CERIOTTI[10] procedure for the estimation of DNA was also employed.

The estimation of radioactivity was carried out in a Nuclear Chicago scaling unit and automatic sample changer fitted with a windowless gas flow counter.

RESULTS

The degradation of DNA with DNase II and 3′-phosphodiesterase liberates the 4 deoxyribonucleoside-3′-monophosphates and a terminal nucleoside from the deoxyribonucleoside end of the DNA chain as shown in Fig. 1. When [^{3}H]DNA prepared in the usual manner was degraded in this way, the major proportion of the radioactivity was recovered in the thymidine-3′-monophosphate and only a small proportion in TDR (Table I). If the [^{3}H]TDR had been incorporated only as a single terminal nucleotide on a pre-existing polynucleotide chain as in Fig. 2 (a) all the radioactivity would have been recovered in the isolated TDR. The occurrence of such a high proportion of the radioactivity in the thymidylic acid indicates that most of the TDR had been incorporated into non-terminal positions on the polynucleotide chain.

The action of 5′-phosphodiesterase on DNA proceeds[7] by the removal of nucleoside-5′-monophosphates in a stepwise fashion from the nucleoside end of the polynucleotide as shown in Fig. 3. In the experiment illustrated in Fig. 4, the release of acid-soluble

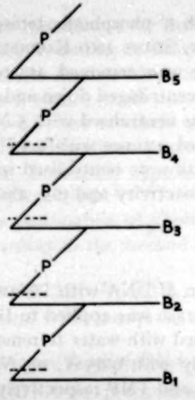

Fig. 1. Degradation of a DNA chain by DNase II and 3′-spleen diesterase.

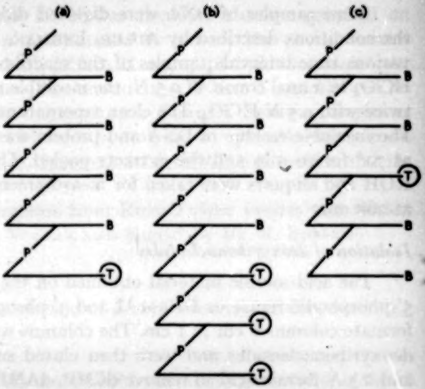

Fig. 2. Possible locations of [³H]TDR incorporated into DNA (see text).

TABLE I

THE RECOVERY OF RADIOACTIVITY IN THYMIDINE-3′-MONOPHOSPHATE (TMP-3′) AND IN THYMIDINE AFTER THE DIGESTION OF [³H]DNA WITH DNASE II AND 3′-PHOSPHODIESTERASE

	Total counts/min	*Per cent total DNA counts/min*	*Per cent acid-soluble counts/min*
DNA	437,500	100	—
TMP-3′	277,000	62	91
TDR*	27,600	6	9

* This was essentially the total nucleoside fraction which was not absorbed by the Dowex-1 formate column.

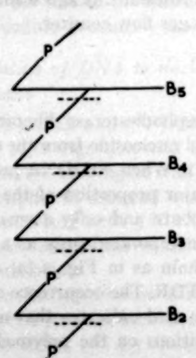

Fig. 3. Degradation of a DNA chain by 5′-venom diesterase.

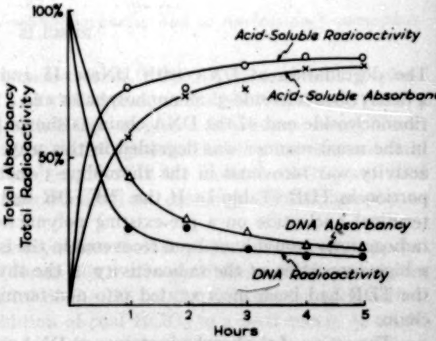

Fig. 4. Release of radioactivity and acid-soluble materials absorbing u.v. light at 268 mμ on the digestion of [³H]DNA with 5′-venom diesterase.

radioactivity and u.v. absorbing material from [^{3}H]DNA was studied during its digestion with 5′-phosphodiesterase. The results show that the release of u.v. absorbing material and radioactivity can be closely correlated and that even after 5-h digestion when only 19% of the DNA remains acid-insoluble, it retains 17% of its original radioactivity. If the [^{3}H]TDR had been incorporated into DNA at the ends of the chain as shown in Fig. 2 (a and b), digestion with 5′-phosphodiesterase would have been expected to release all of the radioactivity at a very early stage in the process before a large proportion of the DNA had been rendered acid-soluble. Since this was not observed, it can be concluded that the [^{3}H]TDR has been incorporated into the polynucleotide chain in a random fashion as illustrated in Fig. 2 (c) and is not attached to the end of the chain as single or multiple additions as in Fig. 2 (a and b).

Further support for this conclusion comes from the results shown in Table II which illustrate the distribution of activity amongst the 4 deoxynucleoside-3′-monophosphates isolated after the degradation of DNA which had been labelled by the incorporation of either [^{32}P]dCMP or [^{32}P]TMP. Since the radioactivity is approx. evenly distributed amongst the 4 deoxyribonucleoside-3′-monophosphates, it is clear that the incorporated dCMP or TMP must have been introduced into the DNA in a random fashion so far as its relationships with the next nucleotide in the chain are concerned. These results can be interpreted either as the completely random incorporation of the labelled nucleotide into a new portion of a polynucleotide chain or as the addition of a single nucleotide unit to the ends of polynucleotide chains terminating randomly. This second type of incorporation has already been excluded by the results already described but it can also be excluded on the grounds of the amount of nucleotide incorporated in relation to the amount of DNA present. In Table III the total quantity of [^{32}P]dCMP or TMP incorporated into the DNA has been calculated. This represents about 15% of the available nucleotide in the case

TABLE II

THE DISTRIBUTION OF RADIOACTIVITY IN THE 4 DEOXYRIBONUCLEOSIDE-3′-MONOPHOSPHATES OF DNA AFTER INCORPORATION OF DEOXYCYTIDINE-5′-PHOSPHATE OR THYMIDINE-5′-PHOSPHATE CONTAINING ^{32}P

Nucleotide incorporated	*Per cent total counts/min recovered in*			
	dCMP	*dAMP*	*dGMP*	*TMP*
[^{32}P]dCMP	27.9	22.3	31.0	18.8
[^{32}P]TMP	34.3	18.7	27.0	20.0

TABLE III

THE INCORPORATION OF [^{32}P]dCMP AND [^{32}P]TMP INTO DNA

	Nucleotide incorporated	
	[^{32}P]dCMP	*[^{32}P]TMP*
Counts/min incubated	225,000	358,000
Total counts/min isolated	35,400	18,500
Per cent incorporation	15.7	5.2
μmoles labelled nucleotide incubated	5	5
μmoles nucleotide incorporated	0.79	0.26

of dCMP and 5 % in the case of TMP. Table IV shows the amount of DNA present in the reaction mixture calculated in terms of µmole DNA-P and also the amount of dCMP incorporated. From these data it is apparent that if the dCMP had been incorporated only as a single terminal nucleotide it would be necessary to postulate that the polynucleotide chain contained only 30 nucleotide residues corresponding to a mol. wt. of less than 10,000. In fact, the DNA added to the reaction mixture initially was a highly polymerised preparation, and since we have been able to detect only very feeble DNase activity in the ascites tumour cell extracts, it seems unlikely that the DNA is seriously degraded during the course of the incubation.

TABLE IV

INCORPORATION OF [^{32}P]dCMP INTO DNA

µmole dCMP incorporated	0.79
µmoles DNA-P present in reaction mixture	24.2
Ratio µmoles DNA-P/µmole dCMP	30

The conclusion reached from these experiments, therefore, is that the thymidine and deoxyribonucleotides incorporated in this system are randomly inserted into a completely new portion of a polynucleotide chain and this has led us to experiments designed to demonstrate net synthesis of DNA.

Net synthesis of DNA has been established when the ascites cell extract is incubated with the usual additions together with all 4 deoxyribonucleoside-5′-monophosphates. Table V shows an increase in amount of DNA present of 81 %, corresponding to the synthesis of 21 µg of polynucleotide in 1 ml reaction mixture. Similar synthesis of DNA has also been observed with extracts containing all four deoxyribonucleoside-5′-triphosphates but without ATP (Table VI). Both dialysed and undialysed preparations of the extract were found to be capable of polynucleotide synthesis.

TABLE V

SYNTHESIS OF POLYNUCLEOTIDE BY SOLUBLE EXTRACTS OF EHRLICH ASCITES TUMOUR CELLS

The reaction mixture contained 100 µmoles tris buffer pH 7.9, 0.25 µmole DPN, 5 µmoles $MgCl_2$, 25 µg ascites DNA and 0.7 ml ascites cell extract in a total vol. of 1 ml. Incubation time: 4 h. Figures in parenthesis represent the number of determinations.

Additions	*Total DNA/tube µg*
Nil	26.2 (4)
0.5 µmole each of dAMP, dGMP, dCMP and TMP + 10 µmoles ATP	47.7 (4)

DISCUSSION

The results presented in this paper indicate that in the system used, precursors such as [^{3}H]TDR and [^{32}P]dCMP and [^{32}P]TMP are incorporated into new portions of polynucleotide chains and suggest that the incorporation of these precursors is a

TABLE VI

SYNTHESIS OF POLYNUCLEOTIDE BY SOLUBLE EXTRACTS OF EHRLICH ASCITES TUMOUR CELLS

The reaction mixture was as in Table V. Incubation time, 4 h. Figures in parentheses represent the number of determinations.

Additions	*Total DNA/tube* µg
Nil	22.2 (4)
0.3 µmole each of dATP, dGTP, dCTP and TPP	40.6 (4)
As above, but with dialysed extract	38.9 (4)

measure of polynucleotide synthesis. This conclusion is confirmed by the demonstration of net synthesis of polynucleotide when all 4 deoxyribonucleoside-5′-monophosphates and ATP or all 4 deoxyribonucleoside-5′-triphosphates are present.

The ability of the system to use deoxyribonucleosides and deoxyribonucleotides for polynucleotide synthesis implies that crude ascites extracts are capable of phosphorylating these compounds to the corresponding polyphosphates[8]. Since the deoxyribonucleoside monophosphates and ATP can be replaced by the corresponding triphosphates in the synthesis of polynucleotide, it seems probable that in this system the immediate precursors of the polynucleotide molecule are the 4 deoxyribonucleoside-5′-triphosphates. This conclusion is supported by the observation that the incorporation of [^{3}H]TDR is strongly inhibited by inorganic pyrophosphate[2]. Similar inhibition by pyrophosphate, etc., has been described for incorporation of [^{3}H]TTP into DNA by an enzyme system for regenerating rat liver[11] and for incorporation of labelled triphosphates into DNA by a purified enzyme from *E. coli*[12, 13].

There is a striking difference between the system that we have described for [^{3}H]TDR incorporation into DNA and the observation of ADLER *et al.*[7] of the incorporation of a single deoxyribonucleoside-5′-triphosphate into DNA by a purified enzyme preparation from *E. coli*. With ascites extracts and [^{3}H]TDR or [^{32}P]-dCMP or [^{32}P]TMP as the only added nucleoside or nucleotide, the incorporation has been shown to represent the formation of a new portion of a polynucleotide chain. In these circumstances the other 3 deoxyribonucleosides or deoxyribonucleotides must have been present in the incubation mixture. If the assumption is made that approx. equimolecular amounts of TDR and the other 3 deoxyribonucleotides are incorporated, about 0.2 to 0.5 mµmole of dCMP, dAMP and dGMP or the corresponding deoxynucleosides would have to be present in the reaction mixture to account for the extent of [^{3}H]TDR incorporation observed. When [^{32}P]dCMP or [^{32}P]TMP was used less than 2 µmoles of nucleotide was incorporated necessitating the presence of not more than comparable amounts of the other nucleosides or nucleotides. These quantities of deoxyribonucleotide materials might well have been present in the cellular extract or could easily have been formed as the result of a small degree of DNA breakdown during the incubation. The liberation of µmolar quantities of deoxyribonucleotides would correspond to the breakdown of less than 4 % of the DNA present. On the other hand, ADLER *et al.*[7] observed that when only one deoxyribonucleoside-5′-triphosphate was added to their purified enzyme system from *E. coli* the nucleotide became attached to the existing polynucleotide chain as single or multiple terminal additions. This observation might well be expected since, in the purified system used by these workers, intrinsic deoxyribonucleoside-5′-triphosphates would not be present and the purification of the polymerase would almost certainly result in removal of the ability to phosphorylate deoxyribonucleoside-5′-monophosphates. Even if any such monophosphates were formed during the incubation they would not be available for polynucleotide synthesis.

The observation that net synthesis of polynucleotide is obtained with dialysed ascites cell extract and the 4 deoxyribonucleoside triphosphates is particularly interesting, since we have previously found that the dialysed extract does not catalyse the incorporation of [^{3}H]TDR into DNA[2]. The explanation of this discrepancy has been shown by our colleague Dr. DEBOV to be due to the almost complete failure of the dialysed extract to phosphorylate TDR to TTP.

It may be concluded that the system described above for the synthesis of polynucleotide by soluble extracts of Ehrlich ascites cells resembles in many respects that described by LEHMAN, BESSMAN, SIMMS AND KORNBERG[12], BESSMAN, LEHMAN, SIMMS AND KORNBERG[13], and ADLER *et al.*[7] for purified enzyme preparations from *E. coli*. The extent of polynucleotide synthesis obtained with the mammalian cell extracts has so far been much smaller than that found by KORNBERG *et al.* A similar system in extracts of regenerating rat liver has been described by BOLLUM AND POTTER[14] and by MANTSAVINOS AND CANELLAKIS[15].

ACKNOWLEDGEMENTS

We should like to thank Mr. C. McLEOD and Mr. B. HIGGINS for skilled technical assistance. This work has been aided by grants from the British Empire Cancer Campaign, The Jane Coffin Childs Memorial Fund for Medical Research, and The Anna Fuller Fund, all of which are gratefully acknowledged.

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MECHANISM OF ACTION OF 6-AZACYTOSINE IN BACTERIA

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(Received April 8th, 1959)

SUMMARY

The growth of *E. coli* B in a medium containing 6-azacytosine resulted in the accumulation of 6-azauracil, 6-azauridine, 6-azacytidine and orotic acid.

6-Azacytosine and cytidine are readily deaminated by whole cells of *E. coli*, *P. vulgaris*, and by acetone preparations of *P. vulgaris*. Although cytosine is readily deaminated by whole cells of *P. vulgaris*, acetone preparations are unable to perform this reaction.

The ability of 6-azacytosine to inhibit these organisms may be correlated with the capacity of these organisms to deaminate the analogue. This suggests that 6-azauracil is the principle bacteriostatic substance.

INTRODUCTION

The possibility that the 1,2,4-triazine analogues of the pyrimidines might be suitable antagonists of the natural pyrimidines was first investigated in connection with studies on 2,4-diaminopyrimidines[1]. These studies culminated in the syntheses by new routes of 3,5-dihydroxy-1,2,4-triazine (6-azauracil), the 6-methyl derivative (6-azathymine), and 3-hydroxy-5-amino-1,2,4-triazine (6-azacytosine)[2]. Independently, in 1956, BARLOW AND WELCH reported the synthesis of 6-azauracil[3]. Both 6-azathymine[4,5] and 6-azauracil[6] had been previously prepared by somewhat less productive methods.

The mechanisms of action of both 6-azathymine[7–9] and 6-azauracil[10–17] have since been studied rather intensively. However, little is known about 6-azacytosine. Although less effective than 6-azauracil as an inhibitor of *Lactobacillus casei*, the cytosine analogue demonstrated greater potency against the mouse mammary Adenocarcinoma 755[18], and against mouse Sarcoma 180[19]. These results prompted a more extensive investigation of the mechanism of action of 6-azacytosine.

Preliminary data demonstrated the presence of orotic acid, azauracil, azauridine, and azacytidine in the medium when *E. coli* B was grown in the presence of 6-azacytosine. This suggested that 6-azauracil, resulting from deamination of 6-azacytosine, was the actual bacteriostatic agent. The data presented in this report lend further credence to this view.

EXPERIMENTAL

The experiments with *Lactobacillus casei* (ATCC 7469) were carried out as previously described, in OFA medium[20]. This medium contains 0.05 mg of folic acid per ml, which is sufficient for half-maximal growth in the usual 3-day assay period. Growth was estimated by titration with 0.1 *N* sodium hydroxide.

Escherichia coli B was grown on a modified C medium[21] (0.01 % magnesium sulfate was substituted for the magnesium chloride and sodium sulfate requirements), supplemented by the addition of 0.5 % glucose. *Proteus vulgaris* was grown on a basal medium consisting of the following: 0.3 % potassium dihydrogen phosphate, 0.7 % dibasic potassium phosphate, 0.5 % ammonium sulfate, 0.01 % magnesium sulfate, 0.25 % dextrose, 0.01 % L-cystine, 20 μg calcium pantothenate/100 ml medium, 20 μg nicotinic acid/100 ml, and 0.06 μg biotin/100 ml. The pH was adjusted to 7.3 with 10 % potassium hydroxide. A mutant of *P. vulgaris*, resistant to 6-azauracil (*P.v.*/AzU) was selected and cultured in the presence of the drug (300 μg/ml). Growth of these organisms was estimated by measurements of turbidity with a Lumetron Model 403-E photoelectric colorimeter. In addition, the organisms were grown in 2-l batches, the cells were harvested, and acetone powders were prepared at —10°.

In an investigation of the metabolism of 6-azacytosine, *E. coli* B was grown on a medium containing the M-9 mixture of inorganic salts[21], supplemented by the addition of 0.3 % glucose. 6-Azacytosine (10^{-3} *M*) was added to cultures of *E. coli* at the beginning of the logarithmic phase and the cells were harvested 4.5 h later. After centrifugation, the supernatant was treated with activated charcoal to remove the nucleic acid metabolites. The latter were then desorbed from the norite by copious quantities of ammoniacal ethanol (95 % ethanol saturated with ammonia at 0°). Following concentration of these extracts under reduced pressure, the metabolites were separated by ascending paper chromatography in 86 % *n*-butanol, and in *sec*-butanol–hydrochloric acid–water (43:11:9). A portion of the solid material obtained upon further evaporation was subjected to chromatography on a column of cellulose powder with 86 % *n*-butanol as the eluent. The u.v. spectra, in water at pH 1 and at pH 11, served as the basis for the identification of the metabolites.

Whole cells of *P. vulgaris*, *E. coli* and suspensions of the bacterial acetone preparations were incubated with equimolar quantities of azacytosine, cytosine, or cytidine in 0.1 *M* phosphate buffer, pH 7.0, for 3 h at 37°, in a Dubnoff shaking apparatus. The details of the various experiments are presented in the legends of the Tables and Figures. The different absorption maxima, at pH 12, of azacytosine, cytosine and of their deaminated products, azauracil and uracil, served as the basis for analysis. The u.v. absorption spectra of cytidine and of its product of deamination, uridine, were determined at pH 1. The appropriate u.v. absorption data for absorption of the pyrimidines, nucleosides and their analogues are presented in Table I. Since the values for the u.v. absorption spectrum of 6-azacytosine were reported incorrectly[8] the details of its spectrum are presented in Fig. 1.

RESULTS

The effects of 6-azauracil and 6-azacytosine upon the growth of *L. casei*, *P. vulgaris* and *E. coli* are tabulated in Table II. *L. casei* is the least sensitive to the azapyrimidines. At lower concentrations, 6-azacytosine is less than one-tenth as effective as 6-azauracil in the inhibition of *L. casei*, but the two analogues are nearly equivalent for *P. vulgaris*. A comparison of the growth obtained with the wild strain with that of the *P.v.*/AzU strain indicates that the latter is cross-resistant to 6-azacytosine.

TABLE I

OPTICAL PROPERTIES OF VARIOUS CYTOSINE DERIVATIVES AND THEIR DEAMINATION PRODUCTS

	pH	*Absorption maximum* *mμ*	*Millimolar extinction coefficient*
Cytosine	12	270	6.05
Uracil	12	285	6.20
Cytidine	2	280	13.3
Uridine	2	262	10.1
Azacytosine	12	295	5.15
Azauracil	12	250	5.21

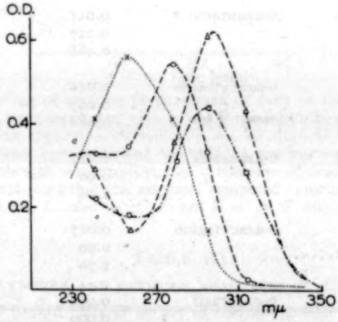

Fig. 1. U.v. absorption spectrum of 6-azacytosine, ○ at pH 1.0, × in H_2O, □ at pH 12.0 and △ at pH 13.0. The curves in the range pH 3.5 to pH 10.6 are almost identical with that for water.

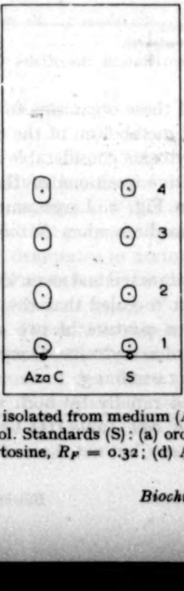

Fig. 2. Chromatogram of metabolites isolated from medium (AzaC) containing azacytosine-treated *E. coli* B, developed in 86% *n*-butanol. Standards (S): (a) orotic acid, $R_F = 0.03$; (b) Azauridine, $R_F = 0.21$; (c) Azacytosine, $R_F = 0.32$; (d) Azauracil, $R_F = 0.50$.

TABLE II

EFFECT OF 6-AZAPYRIMIDINES UPON THE GROWTH OF *L. casei*, *E. coli*, AND *P. vulgaris*

Organism	*Inhibitor*	*Concentration µmoles/ml*	*% decrease in parameter***
L. casei	6-azauracil	0.011	63
		0.033	87
		0.050	95
	6-azacytosine	0.189	68
		0.275	68
		0.900	93
		2.70	95
P. vulgaris	6-azauracil	0.011	77
		0.027	85
		0.088	94
	6-azacytosine	0.011	82
		0.027	85
		0.088	95
P.v./AzU*	6-azauracil	0.027	1
		0.90	4
		2.70	19
	6-azacytosine	0.027	2
		0.90	4
		2.70	26
E. coli	6-azauracil	0.038	1
		0.090	70
		0.90	92
	6-azacytosine	0.090	8
		0.90	57
		2.70	83

* Azauracil-resistant strain of *P. vulgaris*.
** *L. casei* growth was measured by titration, the others were estimated turbidimetrically.

The different sensitivities of these organisms to the 6-azapyrimidines prompted an investigation of the bacterial metabolism of the 6-azacytosine. When *E. coli* was grown in the presence of 6-azacytosine considerable amounts of orotic acid accumulated in the medium. The relative positions of the pyrimidine metabolites after chromatography are depicted in Fig. 2. Large amounts of orotic acid have been reported to accumulate in the medium when *E. coli* was grown in the presence of azauracil[14, 17].

In addition, the presence of azauracil and azauridine in the medium was observed. U.V. spectra at pH 1 and pH 11 revealed that the spot with the R_F value of 0.21 in 86 % butanol, however, was a mixture of two substances, azauridine and azacytidine. The amounts in µmoles of azacytosine, azacytidine, azauracil, and azauridine present, were in the ratio of 1.5:1.0:2.6:1.3.

6-Azacytosine is deaminated rapidly by both whole cell and acetone powder preparations of *P. vulgaris* and *E. coli* (Table III). Cytosine is deaminated at a comparable rate by the cells of *P. vulgaris*, but not those of *E. coli*, and both acetone prepa-

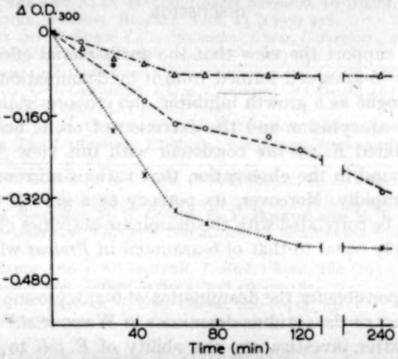

Fig. 3. Suspensions of 20 mg of acetone preparations of each of the three organisms in 0.1 *M* phosphate buffer, pH 7.0, were incubated with 17.9 *μM* of azacytosine in a final volume of 3.0 ml, at 37° in a Dubnoff shaking apparatus. Periodically, 0.1-ml aliquots were withdrawn, introduced into 2.9 ml of 0.01 *N* sodium hydroxide and the O.D. at 300 mμ was determined. The decrease in extinction at this wavelength was employed as a measure of deamination of the pyrimidine. The extinction of a control (omitting the enzyme) remained constant during the course of the experiment. *L. casei* = △, *E. coli* B = ○, *P. vulgaris* = ×.

TABLE III

DEAMINATION OF CYTOSINE AND CYTOSINE ANALOGUES BY *E. coli* AND *P. vulgaris*

Suspensions (0.008 ml of packed cells or 20 mg of acetone powder in 1.0 ml 0.1 *M* phosphate buffer, pH 7.0) were mixed with 2 ml of an aqueous solution containing 17.9 μmoles of pyrimidine and incubated at 37°, for 3 h in a Dubnoff shaking apparatus. At the end of this time, an aliquot was treated as described in the EXPERIMENTAL section.

Substrate	*% deamination*			
	E. coli cells	*P. vulgaris cells*	*E. coli acetone powder*	*P. vulgaris acetone powder*
Azacytosine	100	100	100	100
Cytosine	17	93	16	5
Cytidine	78	87	18	100

rations are relatively inactive. Cytidine, on the other hand, is rapidly deaminated by both of the whole cell preparations, and this activity is retained in the acetone powder of *P. vulgaris*, but lost in that prepared from *E. coli*. WANG *et al.*[22] previously have shown the loss of cytidine deaminase in acetone preparations of *E. coli* E26.

The rates of deamination of azacytosine by the acetone preparations of *E. coli*, *P. vulgaris*, and *L. casei* are depicted in Fig. 3. *P. vulgaris* is the most active in this respect; *L. casei*, the least active. The relationship between the amount of deamination and the time of reaction is linear for the first 60 min. This phase of the curve may serve as a measure of the amount of deaminase present within the organism. The slopes of the curves for *L. casei*, *E. coli*, and *P. vulgaris* are 1.3, 2.8 and 5.6, respectively, and at 60 min correspond to 14.4 %, 32.6 % and 64.2 % deamination of azacytosine, respectively.

DISCUSSION

The present results support the view that the antibacterial effects of 6-azacytosine are due primarily to 6-azauracil formed from it by deamination. The lesser activity of the cytosine analogue as a growth inhibitor, the cross-resistance of the azauracil-resistant *Proteus* to azacytosine and the excretion of orotic acid into the medium by azacytosine-inhibited *E. coli* are consistent with this view. More definitive and direct evidence is found in the observation that various microorganisms deaminate the antimetabolite rapidly. Moreover, its potency as a growth inhibitor of various bacterial species can be correlated with the deaminase activities of the latter, reaching a value approximately equal to that of 6-azauracil in *Proteus* which has the highest deaminase activity.

The enzyme responsible for the deamination of 6-azacytosine is apparently stable to acetone, in contrast to the cytidine deaminase of WANG *et al.*[22]. In agreement with the results of the latter investigators, the ability of *E. coli* to deaminate cytidine was almost totally lost in an acetone powder, although in *P. vulgaris* deamination of this nucleoside proceeded as efficiently in acetone preparations as in whole cells.

The abilities of various tissues and microorganisms to deaminate cytosine and its derivatives have been described in the literature. The deamination of cytidine was observed in crude extracts of liver[23, 24], in mouse kidney[25], and the blood of fowl, but not with mammalian blood[26]. KREAM AND CHARGAFF[27] reported cytosine deaminase activity in brewer's yeast and in *E. coli var. communior*. Employing a different strain of *E. coli* (*E. coli* 15 or *E. coli* E26), WANG *et al.*[22] were able to detect only cytidine deaminase activity, while PAEGE AND SCHLENK[28], using *E. coli*, *A. aerogenes* or *M. lysodeikticus* observed deamination, not only of pyrimidine nucleosides, but also of nucleotides. The deamination of cytosine and 5-methylcytosine has also been observed in various strains of Corynebacterium and Mycobacterium[29]. It is not possible, on the basis of the present results, to identify the azacytosine deaminase definitely with either cytosine or cytidine deaminase.

While the results presented herein suggest that the antibacterial effects of 6-azacytosine are due to the formation of 6-azauracil, deamination cannot account for its antitumor effects since it is at least 10-fold more potent than 6-azauracil as a tumor inhibitor[17, 18].

ACKNOWLEDGEMENT

The authors wish to acknowledge the advice and material help rendered by Dr. R. C. WOOD.

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STUDIES ON THE NUCLEOSIDE PHOSPHOTRANSFERASE OF CARROT

I. PARTIAL PURIFICATION OF THE NUCLEOSIDE PHOSPHOTRANSFERASE

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(Received April 2nd, 1959)

SUMMARY

The partial purification of the nucleoside phosphotransferase of carrot is described. The procedures, which entailed precipitation with acetone, fractionation with ammonium sulfate, and treatment with zinc ions in the presence of ethanol and with calcium phosphate gel, afforded preparations whose specific transferase activity was 40 to 100 times that of the initial extract. The enzyme specimens also exhibited phosphatase activity which could, however, be specifically suppressed by copper or zinc ions. In this manner, good yields of 5′ nucleotides through the transfer of phosphate from phenylphosphate or 5′ adenylic acid to an acceptor nucleoside could be achieved. The acceptor specificity and other properties of the enzyme preparations are also discussed.

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INTRODUCTION

Nucleoside phosphotransferases were discovered a few years ago[1] and studied in some detail with respect to their specificities, as they apply to donor, acceptor and structure of product[2], and also to their distribution in different cells at various stages of growth[3]. They are a group of enzymes capable of effecting the transfer of esterified phosphoric acid to a nucleoside, thus producing a variety of nucleotides. The physiological importance of the nucleoside phosphotransferases, which has been surveyed briefly[4], has, in 2 recent reviews, been in turn affirmed[5] and doubted[6]. We have, in any event, continued the study of this interesting group of transfer enzymes, limiting ourselves in the present series of papers to the nucleoside phosphotransferase of the carrot root. Some of the results have formed the substance of a preliminary communication[7]. For the investigation of the purification of the enzyme and the distinction between hydrolytic and transfer activities, which are being discussed here, plant tissues appeared preferable to animal cells, since—in contrast to the latter—they transfer the phosphoryl group only to the 5′ positions of the acceptor nucleoside[2,3]. Carrots were chosen as a convenient source on the basis of a preliminary survey though other plant material may have served as well[3].

The study of the nucleoside phosphotransferases has so far met with difficulties, since the crude enzyme preparations hitherto available also exhibited strong phosphatase activity. Both the organic phosphate donors and the products formed by phosphorylation were, therefore, decomposed rapidly; and it was almost impossible to answer such fundamental questions as the following: Is the hydrolysis of phosphoric acid esters only a special instance of the transfer of organically bound phosphate groups to hydroxy compounds; in other words, do all acid phosphatase molecules possess transfer activity? Do the transfer enzymes also act as phosphomonoesterases? Or are there specific enzymes whose affinity towards the nucleoside acceptor is so great as to outweigh their ability to transfer the esterified phosphoric acid to water? In an attempt to contribute to a decision on some of these questions, we describe in this paper the partial purification of the carrot enzyme and some of its properties and present, in the following communication, experiments on the separation of nucleoside phosphotransferase and phosphatase activities.

EXPERIMENTAL AND RESULTS

Materials and analytical methods

Commercial preparations of sodium monophenylphosphate (Fisher Scientific Co.) and of uridine (Schwarz Laboratories) were used as donor and acceptor, respectively, in all routine assays. The other nucleosides, employed occasionally as acceptors, also were of commercial origin.

Inorganic phosphorus was determined colorimetrically[8], protein was estimated by the biuret method[9] or, occasionally, by u.v. absorption[10]. The isolation of the enzymically formed nucleotides by means of paper chromatography and their identification and estimation by u.v. spectrophotometry followed, in general, previously published procedures[2].

Enzyme assay

The assays were carried out by incubation at 30° for 1 h in 0.1 *M* acetate buffer of pH 5.2. The total vol. of the assay mixture was 0.2 ml, containing 20 μmoles of sodium phenylphosphate, 4 μmoles of uridine, and an amount of enzyme sufficient to liberate approx. 35 % of the donor P as inorganic phosphate in 1 h. This quantity of enzyme was, as the purification proceeded, determined in preliminary runs with several dilutions of the enzyme. The purpose of this precaution was to maintain fairly uniform conditions for the assay of partially purified transferase preparations that had retained strong phosphatase activity. The preparations were characterized by the previously defined phosphorylation ratio[2], *i.e.*, the molar ratio of phosphate transferred to inorganic phosphate formed, and by the specific activity of the enzyme expressed as μmoles of uridylic acid produced by 1 mg of enzyme protein; both under the standard conditions of assay described above.

Purification

All operations were, unless noted otherwise, carried out at 2 to 4°. Since it was found that more enzyme activity was recovered in the early stages if extracts relatively rich in protein were processed, care was taken to avoid undue dilution. Usually about 1 kg of carrots was extracted in 6 to 8 portions. In a typical experiment with 1000 g of carrots, about 100 g of the root were cleaned, cut into small pieces, and treated with an equal vol. of ice-cold distilled water in a high-speed mincer for 1 to 2 min. The mixture was pressed through cheese cloth and the extract used as the extracting solvent of another portion, which again was passed through gauze, until the entire lot had been processed, each time with the addition of some crushed ice during the grinding. The final extract, after being pressed through several layers of cheese cloth, was made 0.1 *M* with respect to acetate buffer of pH 5.2 by the addition of *M* buffer: its total vol. was about 1000 ml. It is designated as step I in Table I.

Pre-cooled acetone was added slowly, with constant stirring till an acetone concn. of 30 % was reached, the mixture being maintained at 0° or below. The precipitate was entirely devoid of transferase activity. The clear supernatant fluid resulting from centrifugation (15 min at 12,000 × *g*) was brought to an acetone concn. of 70 %. The mixture was shaken mechanically for 30 min and then centrifuged for 15 min at a low speed (450 × *g*). The sediment was extracted with several small portions (3–4 times the volume of the precipitate) of ice-cold distilled water, each time followed by centrifugation, until the extracts, initially reddish-brown, were almost colorless. They were combined and subjected to dialysis against running tap water for 1 h at about 4°. This solution (245 ml) is assayed as step II in Table I.

The solution then was adjusted to 40 % saturation with ammonium sulfate by the slow addition of solid salt with mechanical shaking. The precipitate had only slight activity and was discarded. The supernatant solution was brought to 80 % saturation with ammonium sulfate. The aqueous solution of the resulting precipitate was dialyzed against running water for 1 to 2 h. The final vol. was 43 ml (Step III in Table I).

Ethanol was added slowly to a concn. of 20 %, while the mixture was kept at —5°; and this was followed by the dropwise addition of an ice-cold 0.2 *M* zinc chloride solution till the mixture was 0.005 *M* with respect to Zn^{++}. The precipitate thus produced, which comprised a great deal of the coloring material, did not contain much transferase activity and was discarded. The addition to the supernatant solution of enough cold acetone to depress the water concn. to about 30 % produced an active

TABLE I

PARTIAL PURIFICATION OF NUCLEOSIDE PHOSPHOTRANSFERASE OF CARROT*

Step	Procedure	Volume (ml)	Activity**				Total protein (mg)	Yield(%)		Nucleoside phosphotransferase	
			Phosphatase		Phosphotransferase			Phosphatase	Phospho-transferase	Phosphorylation ratio***	Specific activity §
			Total	per ml	Total	per ml					
I	Crude extract	1000	58,000	58	1350	1.3	6000	100	100	0.02	0.23
II	Pptd. between 30 and 70% acetone	245	40,800	167	1040	4.2	1370	70	77	0.02	0.76
III	Pptd. between 40 and 80% saturation with ammonium sulfate	43	19,200	447	470	11	222	33	35	0.02	2.1
IV	Not pptd. by Zn-ethanol, pptd. by acetone and ammonium sulfate	20	16,200	810	500	25	86	28	37	0.03	5.8
V	Not adsorbed on calcium phosphate gel	21	16,000	762	460	22	67	28	34	0.03	6.9
VI	Dialyzed and lyophilized (60 mg)		9,300		375			16	28	0.04	6.3

* In this experiment 1 kg of carrots was processed.

** The activity is expressed as μmoles of inorganic phosphate (phosphatase) or uridylic acid (phosphotransferase) produced under standard conditions (see text).

*** Molar ratio of phosphate transferred to inorganic phosphate formed.

§ Expressed as μmoles of uridylic acid produced by 1 mg of enzyme protein.

precipitate the aqueous soln. of which was, after dialysis, subjected to a second fractionation with ammonium sulfate, as described before. The fraction precipitating between 40 and 80 % saturation was dissolved in a small amount of ice-cold water and subjected to dialysis. This stage (20 ml of solution, pH 6.0) is referred to as step IV in Table I.

The next step consisted in the removal of inert coloring material by adsorption on calcium phosphate gel[11]. The enzyme solution (about 20 ml) was stirred with 2 ml of the gel (dry wt., 12 mg of $Ca_3(PO_4)_2$) for 1 min at 2° and the mixture immediately centrifuged at 12,000 × *g*. The adsorption was repeated once or twice, until the supernatant solution (21 ml) was pale yellow. This solution (step V in Table I) retained more than 90 % of the transferase activity.

The solution was subjected to dialysis against running water and several changes of ice-cold distilled water for a total of 25 h and the enzyme preparation (60 mg of a white fluff, step VI in Table I) recovered by evaporation in the frozen state in a high vacuum. The yield varied, of course, somewhat with the conditions of the particular experiment. No loss in activity was observed when this preparation was stored at —15° for several months.

A complete balance of this particular experiment is provided in Table I. It will be noticed that, although many of the impurities were removed and the specific activity of the transferase enhanced thirtyfold, the enrichment of the preparation in this enzyme, as compared with phosphatase, was only slight up to step V. Lyophilization, however, had an erratic effect on the phosphatase, occasionally diminishing its activity very considerably, whereas that of the nucleoside phosphotransferase remained essentially unaffected.

The preparation chosen for the example in Table I had an unusually high phosphatase activity, as shown by the low phosphorylation ratio. In other fractionation experiments, this ratio varied in the crude extracts from 0.04 to 0.06*, in the purified transferase preparations from 0.05 to 0.16. The yield in purified transferase fluctuated between 43 and 80 % of the enzyme content of the crude extracts. The specific activity of the final transferase lay between 6.3 and 9.2 and reached, in one case, 34; the enrichment in enzymic activity was between 38 and 96 times when compared with the level of the initial crude extracts.

When the enzyme assay was carried out at 37°, instead of the usual temperature of 30°, the rates of the synthesis of uridylic acid and of the splitting of the donor were both increased, though in both cases the maximal formation of nucleotide was recorded when one half of the phenylphosphate had been split.

As regards the localization of the nucleoside phosphotransferase within the plant cell not much is known except that under our experimental conditions nearly the entire activity was found in the supernatant fluid after centrifugation of the initial extract at 105,000 × *g* for 1 h.

Properties

Acceptor specificity: As was pointed out in discussing the nucleoside phosphotransferase from other sources[2], this group of enzymes appears to be highly specific for nucleosides as acceptors. In the present studies, the crude enzyme (step I) was tested with ethanol, glycerol, D-ribose, D-glucose, and D-fructose without any significant phosphorylation being observed. Similarly, when purified enzyme preparations (step V) were used under standard assay conditions, but with 40 μmoles/ml of the substances to be tested for acceptor function, no phosphorylated products were found with the following compounds: glycerol, *meso*-inositol, D-ribose, D-glucose. These substances, moreover, neither inhibited nor diminished the extent of enzymic phosphate transfer to uridine.

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* It is not unlikely that the relative enzyme content varies with the season. In carrots obtained in spring, the unusually high initial phosphorylation ratio of 0.15 was observed[3].

The conversion of several nucleosides to nucleotides was also studied. With a purified enzyme preparation (step VI) and under standard conditions (100 μmoles phenylphosphate, 20 μmoles nucleoside, 30°) the following quantities of nucleotide (in μmoles/ml/h) were recorded with the nucleosides listed in decreasing order of affinity: cytidine, 1.63; uridine, 0.94; deoxycytidine, 0.82; thymidine, 0.68; inosine, 0.17. Adenosine and deoxyadenosine formed, under the same conditions, only very small amounts of the respective nucleotides.

TABLE II

ACCEPTOR SPECIFICITY; COMPARISON OF CRUDE AND PARTIALLY PURIFIED NUCLEOSIDE PHOSPHOTRANSFERASE OF CARROT

The assays were carried out under standard conditions (100 μmoles phenylphosphate, 20 μmoles acceptor/ml at 30° for 1 h). A constant enzyme concentration was used in each column, with the dilutions so chosen as to minimize phosphatase action. The crude enzymes used in Expts. 1 and 2 were 2 preparations corresponding to step I (Table I). The purified preparation in Expt. 1 corresponded to step IV, that in Expt. 2 to step VI. The ratios indicate the proportions of nucleotide produced by the purified enzyme to that produced by the crude enzyme. Deoxyguanosine and guanosine were also tested, but in a lower concentration owing to their limited solubility. Very similar ratios were observed: deoxyguanosine, 2.0 (Expt. 1), 1.7 (Expt. 2); guanosine, 1.9 (Expt. 1), 1.6 (Expt. 2).

Nucleoside as acceptor	*Nucleotides produced (μmoles/ml/h)*					
	Expt. 1			*Expt. 2*		
	Crude	*Purified*	*Ratio*	*Crude*	*Purified*	*Ratio*
Cytidine	0.63	1.42	2.2	1.1	1.8	1.6
Uridine	0.53	0.97	1.8	0.8	1.35	1.7
Thymidine	0.35	0.75	2.1	0.54	0.88	1.6
Deoxycytidine	0.25	0.54	2.1	0.53	0.95	1.8

In another set of experiments, the efficiencies of crude and purified enzyme preparations with respect to different nucleosides were compared (Table II). As will be seen from the ratios included in this table, fractionation did not affect the respective affinities, as could have been expected, had we been dealing with a mixture of different nucleoside phosphotransferases, each specific for a given acceptor.

Effect of heat: Experiments bearing on this point are summarized in Table III. It may be noted that heating at various temperatures inactivated both phosphatase and nucleoside phosphotransferase to the same extent. Since, as the following paper[12] will show, these activities are attributable to 2 different enzymes, it must be concluded that both are very similar in their sensitivity to heat, if not in other physical properties.

Effect of inorganic phosphate: The influence of inorganic phosphate on the action of a purified sample of nucleoside phosphotransferase (step VI in Table I) is illustrated in Table IV. At the concentrations used, the extent of the production of nucleotide was not affected by the addition of phosphate, but the hydrolytic action of phosphatase was diminished. In previous studies with a crude barley enzyme[2], in which a higher concentration of inorganic phosphate was employed, some effect on the transfer reaction was observed. As was reported before[2, 13], KH_2PO_4, in the absence of donor, produced no nucleotide.

TABLE III

EFFECT OF HEAT ON ACTIVITIES OF PHOSPHATASE AND NUCLEOSIDE PHOSPHOTRANSFERASE

The same partially purified enzyme preparation (step III in Table I), diluted to a protein concentration of 2.7 mg/ml, was used in all experiments, under standard conditions of assay with phenylphosphate and uridine. The enzyme solutions employed in Expts. 2–6 were, after being heated for 2 min, rapidly cooled in an ice bath.

Expt. No.	*Temperature of heating for 2 min*	*(a) Inorg. phosphate liberated, mole % of donor*	*(b) Nucleotide synthesized, mole % of acceptor*	*Ratio of b/a*
1	Unheated	9.4	8.3	0.9
2	40°	7.5	9.0	1.2
3	50°	7.5	9.0	1.2
4	60°	6.5	8.0	1.2
5	70°	4.0	4.0	1.0
6	80°	0.5	0.6	1.2

Selective inhibition of phosphatase action by copper or zinc ions: In all of the following experiments, lyophilized specimens of the partially purified enzyme corresponding to step VI in Table I were employed. In preliminary tests, the effect of numerous cations on the enzyme action was studied. Whereas silver, magnesium, barium, chromium, manganese, cobalt and nickel appeared to have little or no effect on both phosphatase and transferase activities, copper and zinc had a specific inhibiting effect on the phosphatase without affecting the nucleoside phosphotransferase. In Table V, we show a representative experiment with Cu^{++}. It will be noticed that in the presence of the metal almost no hydrolysis of the donor took place during the first hour of incubation or, with lower enzyme concns., even for much longer periods. The conditions prevailing in Expt. 4 (Table V) are probably optimal, if a good yield of

TABLE IV

INFLUENCE OF INORGANIC PHOSPHATE ON ACTION OF NUCLEOSIDE PHOSPHOTRANSFERASE

The concentrations (per ml) used in each experiment were: sodium phenylphosphate, 100 μmoles; uridine, 20 μmoles; lyophilized enzyme preparation (step VI in Table I), 400 μg. Incubation was for 1 h at 30°.

Expt. No.	*Inorg. phosphate added μmoles/ml*	*Inorg. phosphate liberated μmoles/ml*	*Nucleotide synthesized μmoles/ml*
1	0	35	2.2
2	5.5	35	2.2
3	10.5	34	2.1
4	17.5	32	2.1
5	34	28	2.1
6	64	17	2.1

TABLE V

EFFECT OF Cu^{++} ON ACTION OF PHOSPHATASE AND NUCLEOSIDE PHOSPHOTRANSFERASE

The indicated quantities of a lyophilized enzyme preparation (step VI in Table I) and of Cu^{++} were used together with (per ml of 0.1 *M* acetate buffer of pH 5.2) 100 μmoles of sodium phenylphosphate and 20 μmoles of uridine; incubation at 30°.

Expt. No.	*Enzyme μg/ml*	*Copper μg-atom/ml*	*Inorganic phosphate liberated, mole % of donor*			*Nucleotide synthesized, mole % of acceptor*		
			Length of incubation (in hours)					
			1	*2*	*6*	*1*	*2*	*6*
1	500	0	69	81	100	10	1	0
2	500	20	4	52	76	13	18	1
3	250	0	58	76	100	12	7	0
4	250	20	1.5	16	65	10.5	19	10
5	125	0	38	66	80	10	11.5	6.5
6	125	20	0	4	25	9	12	22
7	50	0	17	39	65	5.5	9	8
8	50	20	0	1	4	5.5	9	14.5

nucleotide in a short time and with a moderate amount of enzyme is desired, though a longer incubation with even less enzyme (*e.g.*, Expt. 6) could be considered. The highest yield of uridylic acid observed amounted to 33 mole % of the acceptor uridine. Conditions affording even larger relative quantities of nucleotide will be discussed in the following paper[12].

Another point of interest, noticeable in Table V, is the apparent recovery of the phosphatase from its initial inhibition by Cu^{++}: a recovery proportional to the quantity of enzyme employed. The gradual reversal of the copper effect shows that the metal did not irreversibly inactivate the phosphatase, but acted as an inhibitor by combining with the enzyme or with the enzyme–substrate complex, an equilibrium being established between inhibited and active phosphatase. This shifted in favor of the latter as increasing amounts of copper phosphate were formed by the gradually liberated inorganic phosphate. Addition of phosphate to the enzyme system reversed, in fact, the effect of copper at any time, as did also versene or cysteine.

It may be mentioned that iodoacetate or *p*-chloromercuribenzoate showed no effect on either the phosphatase or the nucleoside phosphotransferase of carrot.

Comparison of two phosphate donors: An experiment comparing the efficiency of phenylphosphate and 5′ adenylic acid as phosphate donors in a partially purified enzyme system (step IV in Table I) is shown in Table VI. In the second instance, the much slower hydrolysis of the donor and the sparing effect on the product of phosphate transfer will be noticed.

Comparison with other nucleoside phosphotransferases: The crude barley enzyme described previously[2] responded to metal inhibition similarly to the carrot preparations. The nucleoside phosphotransferase of rat liver[3], however, exhibited interesting differences.

A partial purification of the rat-liver enzyme was carried out up to a stage corresponding to step III in Table I. Whereas, as mentioned before, Cu^{++} inhibited, reversibly, the carrot phosphatase, but not the transferase, it inactivated, irreversibly, both enzymes of rat liver. Zn^{++}, on the other hand, which inhibited the carrot phos-

TABLE VI

COMPARISON OF TWO PHOSPHATE DONORS

All experiments were carried out in acetate buffer of pH 5.2 with uridine as acceptor (20 µmoles/ml), the indicated amounts of donor and a very dilute enzyme preparation corresponding to step IV (600 µg of protein/ml).

Quantity of donor (µmoles/ml)		*Inorg. phosphate liberated (µmoles/ml)*		*Uridylic acid produced (µmoles/ml)*	
		Hours of incubation at 30°			
Phenylphosphate	***5′ Adenylic acid***	*1*	*24*	*1*	*24*
100		45	100	1	0
	20	1	5.1	0.3	4.9
50	50	28.5	58	0.8	2.7

phatase, was without effect on either the phosphatase or the nucleoside phosphotransferase of rat liver.

DISCUSSION

Although this paper deals almost exclusively with the nucleoside phosphotransferase of carrot, it is not unlikely that the knowledge gained here can be extended to comparable enzyme systems from other plants, *i.e.*, to the group of transferases that limit themselves to the transfer of phosphate to the 5′ position of the acceptor nucleoside[2-4]. We have previously dealt with the very wide distribution of enzymes of this type and their possible physiological significance in the processes attending cellular prolification[3,4]. On the general problem of the mechanisms governing the enzymic transfer of phosphate or phosphoryl groups the excellent review by AXELROD[5] may be consulted. There can be little doubt that the nucleoside phosphotransferase, whose partial purification is described here, shows a very high degree of specificity for nucleosides as acceptors: under comparable conditions, no indication of phosphate transfer to other hydroxy compounds could be found.

The partial purification of the enzyme, which forms the substance of the first part of this communication, provides a relatively convenient procedure for the isolation of transferase preparations, with the specific activity increased fortyfold to a hundredfold of the initial potency. Used in this form and in the presence of copper ion to inhibit the phosphatase, this enzyme system offers an excellent means of preparation of otherwise difficultly accessible 5′ nucleotides.

As can be seen in Table I, the transferase preparations isolated by the partial purification method described here were invariably accompanied by phosphatase. Although occasionally an extensive inactivation of the latter was observed, no definite decision on the identity or difference of the carriers of these 2 enzymic activities could first be reached. The experiments summarized in Table III, in fact, point to a far-reaching similarity of phosphatase and nucleoside phosphotransferase in regard to their response to heat. The action of copper and zinc ions, on the other hand, in inhibiting the phosphatase without affecting the transferase (Table V), could be taken as a strong indication of the nonidentity of the 2 enzymes. In the absence of kinetic data, however, and in view of the general uncertainty concerning the mode of inhibition by metal ions[14], it is preferable to defer the discussion of this problem to the following paper[12] in which a more direct proof of the difference of the 2 enzymes is offered.

As concerns donor specificity, reference may be made to previous reports in which it was shown that, in addition to phenylphosphate, 5′ nucleotides were excellent donors in nucleoside phosphotransferase systems from plants[2,3]. In the present instance, only a few experiments with 5′ adenylic acid as the donor in the partially purified system were carried out. This nucleotide was hydrolyzed at a much lower rate than was phenylphosphate—the contaminating phosphatase appears to act primarily as a 3′ nucleotidase—and had a sparing effect on the product of the phosphate transfer, uridylic acid, which continued to be synthesized for a long period. (See the example in Table VI.)

The problem of acceptor specificity (Table II) is closely connected with the question whether we deal with one transfer enzyme or with several. In previous experiments with crude preparations from barley, no indication of the latter possibility was found[2]. Also in the present study, the comparison of crude and purified enzyme preparations showed no displacement of the affinity spectrum. But it is noteworthy that inosine, and even more markedly adenosine, were poor acceptors of phosphate. Whether the nucleoside phosphotransferases of plants represent a family of specific transfer enzymes remains, for the time being, undecided.

ACKNOWLEDGEMENT

This work was supported by a research grant from the National Science Foundation.

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STUDIES ON THE NUCLEOSIDE PHOSPHOTRANSFERASE OF CARROT

II. SEPARATION OF TRANSFERASE AND PHOSPHATASE ACTIVITIES

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(Received April 2nd, 1959)

SUMMARY

The preparation of nucleoside phosphotransferase, whose isolation and partial purification from carrot extracts are described in the preceding paper, can be divided further. By treatment with Celite, a phosphatase fraction devoid of transferase activity is removed by adsorption, whereas a strong nucleoside phosphotransferase remains unadsorbed. A similar separation of activities is brought about by electrophoresis on filter paper. The transferase fraction retains a small amount of hydrolytic activity which, in contrast to that of the phosphatase, is partially inhibited in the presence of an acceptor nucleoside. Other properties of the enzymes are also discussed.

INTRODUCTION

In the preceding paper[1] the partial purification of the nucleoside phosphotransferase present in carrot extracts was described. Although a considerable enrichment in transferase activity was attained, no clear-cut separation of transferase and phosphatase could be observed. There were, however, indications that the two activities were not attributable to the same protein; and the action of heavy metals in inhibiting the hydrolysis of the phosphate donor without affecting the transfer of phosphate to an acceptor nucleoside supported the postulation of nonidentity.

We present here experiments in which an almost complete separation of the nucleoside phosphotransferase and the phosphatase of carrot was achieved: studies that have formed the substance of a brief note[2]. The latter enzyme was found to be easily adsorbed on diatomaceous earth—in contrast to the transferase—and electrophoretic separation was also feasible.

EXPERIMENTAL

Materials

The enzyme preparations used as the starting material in all separation experiments were partially purified specimens of carrot nucleoside phosphotransferase that had undergone final dialysis and lyophilization, as described as step VI in the preceding paper[1]. The separation presented here was not effective with cruder enzyme solutions.

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Diatomaceous earth was used in the form of Celite Analytical Filter Aid (Johns-Manville).

Procedures

The enzyme assays were performed under the standard conditions mentioned in the preceding paper[1], with sodium phenylphosphate as the donor and ribouridine as the acceptor. When long incubation times were employed, the buffer solutions were protected by 0.001 % of ethyl mercurithiosalicylate. The methods cited before served for the identification of the nucleotides formed by phosphate transfer. Other procedures used will be discussed as they are taken up in the following sections.

RESULTS AND DISCUSSION

Separation of phosphatase and nucleoside phosphotransferase by adsorption

The original material used in the subsequent separation experiments will be referred to as Fraction O. A 0.05 to 0.1 % solution of such a preparation in distilled water was shaken mechanically with diatomaceous earth (20 mg of Celite per mg of protein) at 4° for ½ to 1 h. The adsorbent had previously been washed twice in the centrifuge with distilled water. The mixture was clarified by centrifugation, the sediment washed once or twice with small amounts of distilled water, and the combined supernatant fluids were either used directly for the enzyme experiments or evaporated to dryness in the frozen state in a vacuum. This fraction, which formed a white fluff, will be designated as Fraction T. In a few instances, the solution of Fraction T was treated three more times with Celite, in which case the unadsorbed material will be referred to as T′. In order to recover the portion of the original enzyme preparation that had been adsorbed, the Celite sediment was shaken with 0.1 *M* aqueous ammonium formate (brought to pH 9.5 with ammonia) for 1 h and the extract was, after centrifugation, subjected to dialysis and lyophilization. This material, usually somewhat tan-colored, will be designated as Fraction P.

An approximate estimate of yields may be given. Fraction T amounted to 50 to 60 % of the weight of Fraction O, Fraction P to 25 to 33 %.

A selection of representative experiments is shown in Tables I and II. It will be seen that by the treatment of the partially purified carrot enzyme with diatomaceous earth a phosphatase devoid of transfer activity is removed through adsorption. This phosphatase fraction was strongly inhibited by copper ion; an effect similar to that described previously for the preparations that had not been treated with Celite[1]. (Compare also Table II.) On the other hand, the portion that is not adsorbed displays a strong nucleoside phosphotransferase activity and a relatively weak hydrolytic activity (also depressed by Cu^{++}) demonstrable after a long incubation time. Repeated treatments with the adsorbent have no effect on the transferase activity of Fraction T and fail effectively to remove the residual phosphatase activity carried by this preparation (Table III). The action of Fraction T at different enzyme levels is illustrated in Table IV.

It will be noticed that the amount of nucleotide synthesized within the first hour of incubation is about the same with comparable quantities of the partially purified

TABLE I

SEPARATION OF PHOSPHATASE AND NUCLEOSIDE PHOSPHOTRANSFERASE BY CELITE ADSORPTION*

Expt. No.	*Product*	*Fraction*	*Quantity in assay (μg/ml)*	*Hours of incubation at 30°* *1*	*2.5*	*5*	*20*	*21*	*30*	*48*	*72*
1	Phosphoric acid liberated (mole % of donor)	O	200	51	73	94	100				
		T	100	0	3	8	18				
	Uridylic acid produced (mole % of acceptor)	O	200	9	7	0					
		T	100	10	17	21	25				
2	Phosphoric acid liberated (mole % of donor)	O	250	60	80			100			
		T	125	0	0			14	17	47	80
		P	75	37	63			100			
	Uridylic acid produced (mole % of acceptor)	O	250	7	3			0			
		T	125	6	13			34	37	46	60
		P	75	0	0	0					

* See the text for experimental details. O, original partially purified enzyme preparation from carrots; T, fraction of O not adsorbed on Celite; P, fraction of O adsorbed on Celite. The enzymic assay mixture contained (per ml) 100 μmoles of Na phenylphosphate, 20 μmoles of uridine and the indicated amounts of enzyme in 0.1 *M* acetate buffer of pH 5.2. Portions of the mixtures were analyzed at the stated intervals.

TABLE II

SEPARATION OF PHOSPHATASE AND NUCLEOSIDE PHOSPHOTRANSFERASE BY CELITE ADSORPTION (EXPT. 3)*

Fraction	*Quantity in assay (μg/ml)*	*Inorganic phosphate liberated (mole % of donor)*					*Uridylic acid produced (mole % of acceptor)*			
		Hours of incubation at 30°								
		1	*3*	*6*	*24*	*54*	*1*	*6*	*24*	*54*
O	150	12	26	40	94		9	22	3	
O**	150	0	1	3	70	100	11	23	25	0
T	250	0	4	10	42	88	11	26	31	16
P	250	50	100				0	0	0	0

* See Table I for explanations.

** In this experiment 20 μg-at/ml of Cu^{++} were present. (Compare the preceding paper[1].)

enzyme and of the preparation deprived of phosphatase by adsorptive treatment, though in the first instance more than one half of the donor is decomposed. The further course of events, however, differs very appreciably in the two cases. In the absence of phosphatase, the phosphate transfer continues despite the gradual splitting of the donor during long periods of incubation.

As concerns the peculiar hydrolytic activity of the transferase preparations (Fraction T), which, as has been shown above, becomes apparent in prolonged incubation, the effects of the absence or presence of an acceptor nucleoside are not without interest, especially when compared with the behavior of partially purified enzyme preparations that had not been treated with Celite. In the latter case, the omission of the acceptor (20 μmoles of uridine per ml) had no measurable effect on the extent of

TABLE III

REPEATED TREATMENT OF NUCLEOSIDE PHOSPHOTRANSFERASE WITH CELITE*

Fraction	*Inorganic phosphate liberated (mole % of donor)*				*Uridylic acid produced (mole % of acceptor)*		
	Hours of incubation at 30°						
	1	*2.5*	*6*	*20*	*1*	*2.5*	*6*
O	52	82	100		8.2	6	0
T	< 1	5	17	51	9	17	25
T′	0	1	9	38	11.2	19	26

* The solution of the partially purified (step VI) carrot enzyme containing 250 µg/ml (Fraction O) was treated with Celite once and the supernatant solution (Fraction T) was shaken in succession three more times with fresh portions of the adsorbent, yielding the supernatant solution designated as Fraction T′. Equal portions of the solutions were used in all experiments. Assay conditions as in Table I.

the splitting of the donor (100 µmoles of phenylphosphate per ml). An entirely different picture emerges when the transferring enzyme that had undergone treatment with Celite is tested with varying concentrations of acceptor (Table V). Whereas the extent of nucleoside production is, at high acceptor concentrations, not influenced a great deal by the amount of acceptor available, the latter has a strong inhibiting effect, commensurate with its concentration, on the hydrolysis of the donor. It would appear that the nucleoside phosphotransferase has a much greater affinity for the nucleoside than for water. It may be of interest to mention that from the data given in Table V for the 3 h experiment it can be computed that at a concentration of about 14 µmoles of uridine per ml equal amounts (namely, 2 µmoles) of uridylic acid and inorganic phosphate are produced.

TABLE IV

ACTION OF NUCLEOSIDE PHOSPHOTRANSFERASE AT DIFFERENT ENZYME LEVELS*

Fraction	*Quantity in assay (µg/ml)*	*Inorganic phosphate liberated (mole % of donor)*					*Uridylic acid produced (mole % of acceptor)*				
		Hours of incubation at 30°									
		1	*3.5*	*6*	*24*	*48*	*1*	*3.5*	*6*	*24*	*48*
O	250	42	100				7	0			
T	500	0	6	15	27	94	14	28	32	24	9
	100	0	2	3	10	17	4	9	15	32	38
	50	0	2	2	5	10	2	4	8	26	39
	12.5	0	0	1	2	4	1	2	3	9	16

* See Table I for explanations.

Separation of phosphatase and nucleoside phosphotransferase by paper electrophoresis

Several orienting experiments were performed which demonstrated the feasibility of separating the phosphatase and transferase activities by electrophoresis on filter paper. The results of two experiments, in which partially purified enzyme preparations from carrot corresponding to Fraction O (stage VI in the preceding paper[1]) were

TABLE V

EFFECT OF DIFFERENT URIDINE CONCENTRATIONS ON THE HYDROLYTIC AND TRANSFERASE ACTIVITIES OF NUCLEOSIDE PHOSPHOTRANSFERASE (AFTER TREATMENT WITH CELITE)*

Quantity of uridine (µmoles/ml)	*Inorganic phosphate liberated (µmoles/ml)*					*Uridylic acid produced (µmoles/ml)*			
	Hours of incubation at 30°								
	3	*18*	*27*	*45*	*95*	*3*	*18*	*27*	*45*
35	0	7	15	23	51	2.6	6.1	7.8	8.8
20	1	15	23	39	80	2.1	6.0	7.4	6.6
9	3	21	35	60	95	2.0	2.9	3.2	2.1
4	4	31	50	75	100	1.3	1.3	2.7	1.2
0	6	39	64	100					

* Each experiment employed (per ml) 100 µmoles of phenylphosphate, the indicated quantities of uridine, and 100 µg of transferase (Fraction T).

employed, are shown in Table VI. Two principal bands were seen, one on either side of the origin. The band migrating to the anode was found to contain phosphatase free of transferase; the band moving to the cathode contained the nucleoside phosphotransferase which, in accordance with the findings detailed above, exhibited also a weak hydrolytic activity. Two or three much lighter protein zones were also seen occasionally on the electropherograms, but they were inert enzymically. For the detection of the protein zones, thin guide strips were cut off along the edges of the sheets after the completion of the run and stained with ninhydrin or bromophenol blue. Each located band was then eluted with 1 ml of ice-cold distilled water and equal portions were tested for activity under standard assay conditions (see Table I). Other experimental conditions are given in Tables VI and VII. The latter table reproduces an experiment, performed with very small amounts of enzyme, comparing the electrophoretic properties of the original, partially purified enzyme preparation with those of the two fractions separable by treatment with diatomaceous earth. It will be seen that the transferase (Fraction T), which had remained unadsorbed on Celite,

TABLE VI

ELECTROPHORETIC SEPARATION OF NUCLEOSIDE PHOSPHOTRANSFERASE AND PHOSPHATASE*

Product	*Electrophoretic fraction*	*Expt. 1*		*Expt. 2*		
		Hours of incubation at 30°				
		1	*6*	*3*	*5*	*24*
Phosphoric acid liberated	A	6	17	30	45	100
(mole % of donor)	C	0	0	0	< 1	24
Uridylic acid produced	A	0	0	0	0	0
(mole % of acceptor)	C	3	7	6	11	26

* A solution of the partially purified, lyophilized carrot enzyme (Fraction O, step VI in the preceding paper[1]) was subjected to electrophoresis (Reco model E-800-2) on Whatman No. 1 paper for 3 h, with the use of a supporting electrolyte of 0.1 M acetate buffer (pH 5.2) and of a gradient of 11 V/cm. Circulating ice-cold water was used for cooling. Two principal bands were located at about + 2 cm and — 2 cm from the origin. The eluate of the anodic fraction is designated A, that of the cathodic fraction C. Conditions of enzyme assays as in Table I.

TABLE VII

EFFECT OF CELITE TREATMENT ON ELECTROPHORETIC BEHAVIOR OF NUCLEOSIDE PHOSPHOTRANSFERASE FRACTIONS*

Starting material, fraction	*Product*	*Electrophoretic fraction*	*Hours of incubation at 30°*			
			3	*16*	*24*	*48*
O	Phosphoric acid liberated (mole % of donor)	A	5		32	80
		C		0	0	1
	Uridylic acid produced (mole % of acceptor)	C		4	5	12
T	Phosphoric acid liberated (mole % of donor)	A	0		0	0
		C		0	0	< 1
	Uridylic acid produced (mole % of acceptor)	C		4	6	13
P	Phosphoric acid liberated (mole % of donor)	A	3		20	

* See Table I for the designation of starting materials and the description of assay conditions; see Table VII for conditions of electrophoresis and the designation of electrophoretic fractions. The electrophoresis was performed on a filter paper sheet containing 5 lanes. Lanes 1 and 2 were each charged with Fraction O, lane 3 with Fraction T, lane 4 with Fraction P, lane 5 with Fraction T. After separation was completed, lanes 1 and 5 were cut off and stained to serve as guide strips for the elution of the appropriate areas from lanes 2 to 4.

retained its transferase activity in the cathodic fraction; it also showed a protein zone on the anodic side, but this was inert, in contrast to the corresponding areas from both the original preparation O and Fraction P eluted from the adsorbent.

CONCLUDING REMARKS

The present study has shown that partially purified enzyme preparations from carrot can be separated into a phosphatase entirely devoid of nucleoside phosphotransferase activity and into a nucleoside phosphotransferase. Further work will have to prove whether the slight hydrolytic action exhibited by the latter enzyme is an inherent property of the transferase, as we believe to be the case, or is due to a second phosphatase different in properties from the one removed by adsorption; a decision that will have to await the further purification, and if possible the crystallization, of the enzyme. Recently, strong doubt has been expressed as concerns the physiological importance of the transferases, as they are said to be nonspecific as to donor and acceptor and to require very high concentrations of acceptor[3]. Some of the findings presented here certainly fail to bear this out. It must, however, be pointed out that it is very difficult to decide what is meant by an important enzyme. The attempt to establish an hierarchical order of nature, if it should be undertaken at all, can hardly be based on popularity contests; nor will appraisals that are often made on teleological, and sometimes on aesthetic, grounds carry much conviction. The plain fact of existence surely should outweigh all these considerations. It may, however, be possible to design a test that would evaluate the relative contributions of the several known nucleotide-forming enzyme systems to the synthesis of nucleotides in the living cell.

ACKNOWLEDGEMENT

This work was supported by a research grant from the National Science Foundation.

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HYDROGEN EVOLUTION AND EXCHANGE, AND CONVERSION OF N_2O TO N_2 BY SOYBEAN ROOT NODULES

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(Received April 4th, 1959)

SUMMARY

Soybean root nodules support the evolution of H_2 and an exchange reaction yielding HD from D_2 and endogenous H donors. The evolution of hydrogen is inhibited by N_2 and N_2O but is rather insensitive to CO. The exchange reaction is strongly inhibited by CO and by N_2O but is activated by N_2. N_2O is converted to N_2 by soybean nodules. A working hypothesis to encompass these experimental observations has been proposed.

INTRODUCTION

In earlier studies[1] it was shown by mass spectrometric analysis that excised soybean root nodules evolved H_2. The evolution of H_2 depended upon the presence of O_2. Under the experimental conditions employed, the evolution of H_2 appeared to be considerably more vigorous than the exchange reaction which produced HD from D_2 and H from the cells or medium. The time course of H_2 liberation was quite similar to the time course of N_2 fixation in excised nodules[2], and their ability to evolve H_2 decreased much as did nitrogen fixation with aging of the plants and upon slicing or grinding of the nodules[3].

The evident importance of hydrogen metabolism in nitrogen fixation has prompted an extension of the earlier studies. The current work indicates the influence of N_2, N_2O and CO on H_2 evolution and exchange and discusses preliminary observations on the metabolism of N_2O in excised nodules.

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METHODS

LINCOLN variety soybeans were grown in the field, and LINCOLN and BLACKHAWK varieties were grown in controlled environment chambers. The seeds were treated with a commercial soybean inoculant prior to planting. The soybeans in the controlled environment chambers were grown in fresh vermiculite, and their nutrients were supplied by watering with HOAGLAND AND ARNON's[4] nitrogen-free solution. The chambers provided a cycle of 15 h light and 9 h dark, a light intensity of about 2000 foot-candles, and a temperature of 27°. Field grown soybeans were harvested when 5 to 8 weeks old and soybeans from the chambers when 5 to 6 weeks old. Soil or vermiculite was washed from the roots, and the nodules were removed, washed and weighed.

The procedures for exposure of the nodules to various atmospheres, sampling of the atmospheres and analysis in the mass spectrometer have been described previously[1]. For measurement of the ^{15}N content of N_2, the gas sample bulbs were immersed in liquid air prior to admission of the gas to the mass spectrometer, and the atom percent ^{15}N was calculated both from the ratio of masses 29 to 28 and from the peak heights at masses 28, 29 and 30. When the atom percent ^{15}N in N_2O was measured, the samples were withdrawn into sample bulbs containing Ascarite, to absorb CO_2, and were allowed to stand overnight. The atom percent ^{15}N was calculated from the peak heights at masses 44, 45, and 46.

RESULTS

Evolution of H_2

N_2 inhibits the evolution of H_2 by soybean root nodules. Fig. 1 indicates that 0.1 atm of N_2 inhibits H_2 evolution by about 30% and 0.3 atm inhibits by about 45%; the inhibition was reasonably uniform with time within the experiments. The data of Fig. 1 are representative of several experiments, although in one experiment there was no apparent inhibition by N_2.

Fig. 2 indicates that N_2O also inhibits H_2 evolution from soybean nodules. Its inhibition is more marked than that by N_2, for 0.1 atm inhibits by about 55% and 0.3 atm by about 65% (calculated from the approximate slopes of the curves). In another experiment with soybean nodules, the inhibition was somewhat greater than that shown in Fig. 2, with inhibitions of approx. 67%, 77%, 86% and 91% at pN_2O values of 0.1, 0.3, 0.5 and 0.7 atm, respectively.

The response of H_2 evolution to CO has been erratic. In some experiments, 0.01 atm CO has inhibited H_2 evolution, and in other comparable experiments CO has enhanced H_2 evolution. Until more extensive experiments can be performed at a variety of CO concentrations, it will be inadvisable to draw conclusions regarding the influence of CO on H_2 evolution.

Exchange reaction to form HD

In contrast to its inhibition of H_2 evolution (Fig. 1), N_2 stimulated the formation of HD from D_2 and a hydrogen donor or donors from the medium or nodules. Fig. 3 indicates that very little HD was formed by soybean nodules in the absence of added N_2, but that its production was rather rapid in the presence of 0.1 or 0.3 atm of N_2.

Although N_2 promotes the exchange reaction, N_2O does not. Fig. 4 shows that the formation of HD in the presence of N_2 is inhibited by N_2O. In the same experiment recorded in Fig. 4, flasks were included without N_2. Very limited exchange occurred,

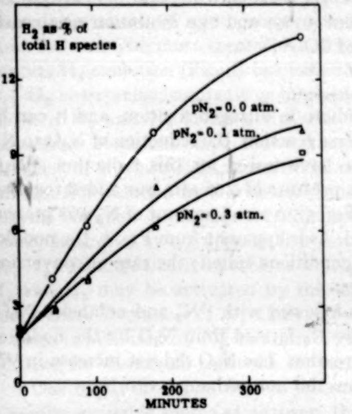

Fig. 1. Inhibition by N_2 of H_2 evolution from soybean root nodules. All reaction vessels contained 0.3 atm D_2, 0.2 atm O_2 and N_2 as indicated; no diluent gas was added. Temp. 30°. H_2 is recorded as % of the total hydrogen species (sum of masses 2, 3 and 4).

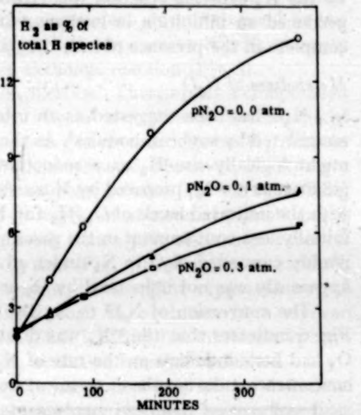

Fig. 2. Inhibition by N_2O of H_2 evolution from soybean root nodules. All reaction vessels contained 0.3 atm D_2, 0.2 atm O_2 and N_2O as indicated; no diluent gas was added. Temp. 30°. The dominant mass 4 peak changes little with time and serves as an internal reference.

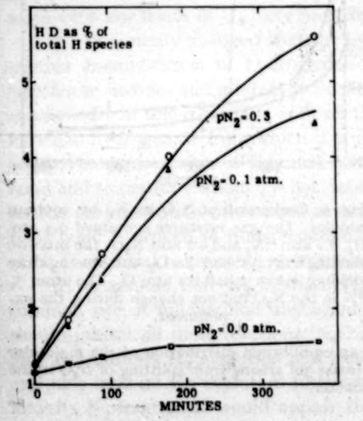

Fig. 3. Enhancement of the exchange reaction by N_2. All reaction vessels contained 0.3 atm D_2, 0.4 atm O_2, and N_2 in atm as indicated; no diluent gas was added. Temp. 30°.

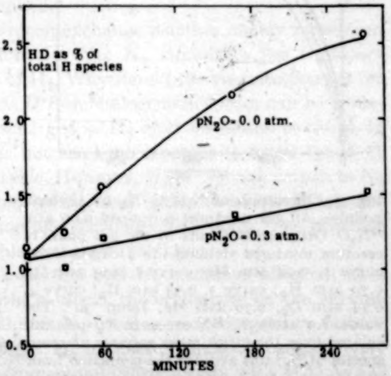

Fig. 4. Inhibition by N_2O of the formation of HD through the exchange reaction. All reaction vessels contained 0.15 atm D_2, 0.25 atm O_2, 0.3 atm N_2 and N_2O as indicated; no diluent gas was added. Temp. 30°.

and any difference between HD formation in the presence and absence of N_2O was within the error of the determinations.

The exchange reaction was clearly sensitive to CO, whereas the response to CO by the H_2 evolving reaction was erratic. Levels between 0.005 and 0.01 atm of CO produced an inhibition in exchange of about 50 %, and the inhibition was nearly complete in the presence of 0.02–0.03 atm of CO.

Metabolism of N_2O

N_2O has been suggested as an intermediate in nitrogen fixation, and it can be assimilated by soybean nodules[5]. As the reverse reaction, the reduction of N_2O to N_2, might logically use H_2 as a reductant, we have tested for this reduction. $^{15}N_2O$ (18.8 atom % ^{15}N; prepared by MOZEN[5]) at a pressure of 0.06 atm was added together with the indicated levels of O_2, H_2 and He (Fig. 5). A small amount of N_2 was present initially as a contaminant in the gases added. As is apparent from Fig. 5, the nodules readily converted N_2O to N_2 under all the conditions tested; the rate of conversion apparently was not influenced by O_2 or H_2.

The conversion of N_2O to N_2 also was followed with $^{15}N_2$ and unlabeled N_2O. Fig. 6 indicates that the $^{15}N_2$ was diluted by N_2 formed from N_2O by the nodules; O_2 had little influence on the rate of N_2 formation. The N_2O did not increase in ^{15}N concentration during the experiment and thus did not exchange with N_2.

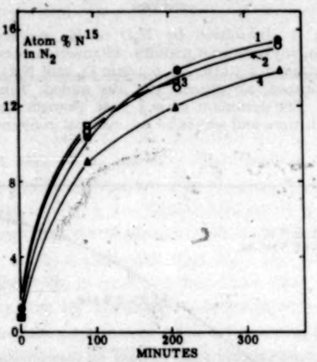

Fig. 5. Conversion of N_2O to N_2 by soybean nodules. All gas mixtures contained 0.06 atm $^{15}N_2O$. Other constituents of the gas phase in reaction mixtures yielding the 4 curves were: curve 1, 0.94 atm He; curve 2, 0.24 atm O_2; 0.70 atm H_2; curve 3, 0.94 atm H_2; curve 4, 0.24 atm O_2, 0.70 atm He. Temp. 30°. The values for atom % ^{15}N excess in N_2 were calculated from the ratios mass 29/mass 28 assuming an equilibrium distribution of the molecular species of N_2; this avoided the confusion from NO (mass 30) arising from splitting of N_2O in the mass spectrometer.

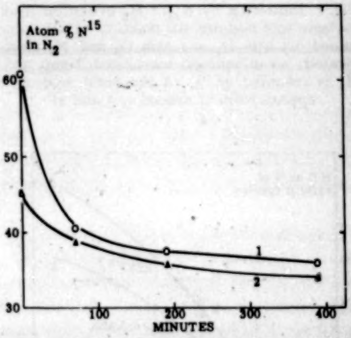

Fig. 6. Conversion of N_2O to N_2 by soybean nodules. The gas mixtures contained 0.3 atm D_2, 0.2 atm $^{15}N_2$ and 0.2 atm N_2O; the mixture yielding curve 1 had no O_2 and the mixture yielding curve 2 had 0.2 atm O_2. The atom % ^{15}N in the N_2O did not change during the experiment.

DISCUSSION

The inhibition of H_2 evolution by N_2 led KAMEN AND GEST[6] to the observation that *Rhodospirillum rubrum* can fix N_2. The long apparent relation between H_2 and N_2

metabolism in nitrogen fixing organisms is reemphasized by the current observations, but the exact nature of the interrelationship still is far from clear.

The nodules of soybeans evolve H_2 at a substantial rate and support an exchange reaction which produces HD from D_2. Although both the evolution and exchange are manifestations of hydrogenase activity, the 2 reactions do not respond in the same fashion. The most spectacular difference is their response to N_2; N_2 strongly inhibits H_2 evolution (Fig. 1) but enhances the exchange reaction (Fig. 3).

H_2 is a specific, competitive inhibitor of N_2 fixation[7]. This implies a competition between H_2 and N_2 at an active enzyme site. Tests[8] with heavy suspensions of *Azotobacter vinelandii* have shown liberation of a gas assumed to be H_2, but no influence of N_2 on this H_2 liberation has been demonstrated. However, N_2 strongly inhibits H_2 evolution by *R. rubrum*[6,9] and from soybean nodules. The nitrogen fixing enzyme complex in nodules is half saturated at a pN_2 of about 0.025 atm[10] and is nearly saturated at 0.1 atm, so it is not surprising that a pN_2 of 0.1 atm supports the H_2 exchange nearly as well as a pN_2 of 0.3 atm (Fig. 3). As suggested earlier[11], N_2 and H_2 may be activated by the same enzyme, a viewpoint supported by the competitive inhibition of N_2 fixation by H_2. Although H_2 evolution from soybean nodules is inhibited by N_2, the data do not establish whether the inhibition is competitive, for the actual substrate supporting H_2 evolution remains undefined.

One might speculate that as hydrogen evolution occurs only in the absence of a readily available source of nitrogen, the evolution may represent an overflow from a sink of reducing compounds normally used to reduce N_2. However, Bregoff and Kamen[12] observed that a mole of ammonia or N_2 prevented release of several moles of H_2 from *R. rubrum*. They concluded that the nitrogenous compounds were acting in a catalytic rather than a stoichiometric fashion to hinder evolution of H_2. Furthermore, the suggestion that H_2, although inhibitory to N_2 fixation, may itself serve as an H donor for reduction of N_2, has never been supported experimentally even when very low levels of H_2 have been supplied.

It is commonly assumed that the hydrogen exchange reaction merely represents another manifestation of hydrogenase activity. Yet N_2 stimulates the exchange reaction in nodules and inhibits evolution of H_2. Why should the two reactions differ so markedly in this regard? It appears that H from endogenous donors can be transferred to hydrogenase from which it is discharged as H_2 after formation of the H–H bond. The readsorption of H_2 must occur, but the back reaction to break the H–H bond and to permit exchange is not detectable. However, H_2 or D_2 may attach to N_2 on the enzyme, or to other sites activated by N_2, and under these conditions the H–H bond is broken so that recombination and exchange may occur. The presence of N_2 is essential to promote an appreciable exchange reaction.

If we assume that N_2 fixation, H_2 evolution and H_2 exchange all occur at a common site, it follows that any agent which blocks the active site on the enzyme should inhibit all these reactions. N_2O inhibits N_2 fixation in a competitive fashion and also inhibits H_2 evolution and exchange. H_2 inhibits N_2 fixation competitively and also inhibits H_2 evolution (inferred from unpublished results with D_2). As CO inhibits N_2 fixation it would appear that it also should decrease H_2 evolution and exchange; actually it strongly inhibits exchange and has a minor effect on H_2 evolution. Unlike H_2 and N_2O, CO is not primarily a competitive inhibitor[13] of N_2 fixation, so it is unnecessary to assume that it blocks the initial step in N_2 fixation.

If it inactivates the N_2 fixation process after the binding and activation of N_2, it could block the H_2 exchange reaction without inhibiting H_2 evolution. If H_2 evolution and exchange are catalyzed at different sites, the differential effect of CO would imply merely a greater affinity of CO for one of the sites.

The experimental results reported can be formulated into the frankly schematic working hypothesis shown in Fig. 7. The scheme provides for competition of N_2, H_2, and N_2O on the native enzyme as well as its acceptance of H (later liberated as H_2) from donors other than H_2. N_2O could form N_2 on the enzyme, but the back reaction would be negligible. CO could block reduction of the enzyme–N_2 complex by direct inactivation or by inhibiting the transfer of H necessary for N_2 reduction. Either mechanism also could inhibit the H_2 exchange reaction. The scheme formulated indicates that the N–N bond is not broken until complete reduction to NH_3 is achieved. Trapping of hydrazine before the final reduction step could yield the products observed by BACH[14].

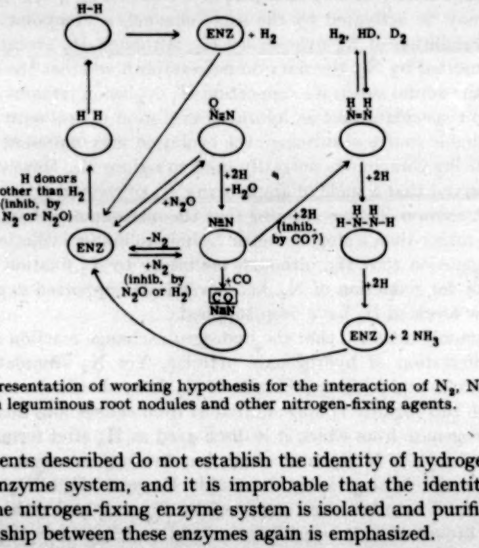

Fig. 7. Schematic presentation of working hypothesis for the interaction of N_2, N_2O, H_2 and CO in leguminous root nodules and other nitrogen-fixing agents.

The experiments described do not establish the identity of hydrogenase and the nitrogen-fixing enzyme system, and it is improbable that the identity will be established until the nitrogen-fixing enzyme system is isolated and purified. However, the close relationship between these enzymes again is emphasized.

The metabolism of N_2O has been intriguing, because this nitrogenous compound is a competitive inhibitor of N_2 fixation and at the same time can be assimilated to a limited extent[5]. In the earlier studies[5], care was taken to remove all N_2 from the N_2O supplied to *A. vinelandii* and to root nodules; ^{15}N from the N_2O was assimilated by the agents. The current work indicates that nodules can convert N_2O to N_2, for $^{15}N_2O$ yielded $^{15}N_2$ and the ^{15}N concentration of $^{15}N_2$ was diluted by N_2 from normal N_2O (Figs. 5 and 6). This suggests that the assimilation of N_2O may be by way of N_2, but that N_2O is not an intermediate in N_2 fixation. Its inability to function as an intermediate was emphasized by the fact, that in the presence of $^{15}N_2$, normal N_2O did not accumulate ^{15}N.

Assimilation of N_2O after an initial conversion to N_2 also is compatible with the earlier observations[15] on the azotobacter that assimilation of ^{15}N from $^{15}N_2O$ is inhibited by H_2 and by N_2. However, if N_2O is assimilated via N_2, one may properly ask why it does not enhance rather than inhibit the exchange reaction. This is probably because its primary action is a competition with N_2 for the active enzyme, and any blocking of N_2 activation at this point will be reflected in a decrease in the exchange reaction. Normally the conversion of N_2O to N_2 must be rather slow and hence rate limiting for activation of the exchange reaction; if this conversion of N_2O to N_2 were rapid, N_2O would not be an effective inhibitor of N_2 fixation. It should be reemphasized that the scheme in Fig. 7 constitutes a working hypothesis only; although it depicts the assimilation of N_2O by way of N_2, certain of the observations suggesting the scheme are not specific for the route recorded but are entirely compatible with assimilation of N_2O in nodules by a path independent of N_2.

ACKNOWLEDGEMENTS

This work was published with the approval of the Director of the Wisconsin Agricultural Experiment Station and was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and by grants from the National Science Foundation and the Rockefeller Foundation.

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THE GROWTH OF ALGAE IN DEUTERIUM OXIDE

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(Received April 6th, 1959)

SUMMARY

Chlorella vulgaris, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* can be grown in 99.6% deuterium oxide media. The rate of growth of the deuterated organisms is great enough to make it possible to use them as a practical source of biosynthesized fully-deuterated compounds.

INTRODUCTION

The behavior of algae in heavy water has been the subject of numerous investigations, most of which were carried out during the 'thirties shortly after the discovery of deuterium; references to the early literature may be found in the review by MOROWITZ AND BROWN[1]. In connection with recent studies on the biological effects of deuterium[2] it appeared desirable to undertake the cultivation of autotrophic organisms in completely deuterated media. Not only do such studies contribute to an understanding of the effects of deuterium on growth, but successful culturing would make possible the biosynthesis of fully deuterated compounds highly interesting both for future metabolic studies and for possible therapeutic applications.

The prospects for successful culturing of algae in deuterium oxide were certainly dubious. Most of the early workers reported that algae showed little or no growth in nutrient media containing more than 75–85% deuterium. The more recent studies of WEINBERGER AND PORTER[3] and of MOSES, HOLM-HANSEN AND CALVIN[4] were in agreement with this conclusion. The latter workers reported that *Chlorella pyrenoidosa* grew very poorly at deuterium levels above 60% even if the algae were serially subcultured into increasing concentrations of deuterium, and their inability to obtain useful growth rates at elevated deuterium levels has been very recently reiterated[5]. It is to be noted that all of these workers allowed only 4 or 5 days for the algae to adjust to the elevated deuterium levels; in our experience this is ordinarily a period far too short for the algae to become adapted or acclimatized to a highly deuterated environment. On the other hand, MEYER[6] stated that "*Chlorella vulgaris* grown in nutrient solutions made with D_2O appeared completely healthy and grew abundantly, and indeed as well as in the control. Heavy water for the object of this investigation is as good as ordinary water". Unfortunately there is some question as to the degree of enrichment of the deuterium oxide employed by MEYER. Although he describes the deuterium oxide he employed as 99.2% D_2O, the specific gravity of water is given as 1.049. This specific gravity actually corresponds to a D_2O concentration of 45%. In any event, our results are in agreement with those reported by MEYER[6] and differ materially from those reported by MOSES, HOLM-HANSEN AND CALVIN[4]. Our experiments indicate that at least 3 green algae will grow and divide in 99.6% deuterium oxide, and that these algae can be grown at a rate such that they become a practical source of fully-deuterated compounds.

MATERIAL AND METHODS

Growth of algae

Three species of green algae, *Chlorella vulgaris*, *Chlorella pyrenoidosa*, and *Scenedesmus obliquus* were used in the experiments described here. The algae were cultured in approx. 125 ml of nutrient solution contained in 250-ml Erlenmeyer flasks which were fitted with inlet and outlet tubes to prevent exchange of deuterium oxide with the water of the atmosphere. Growth took place at 28° under continuous light of an intensity of 600-ft candles (supplied by a fluorescent panel) and with continuous agitation by a mechanical shaker. The algae were supplied a gaseous mixture, at the rate of about 20 ml/min, composed of 95% nitrogen and 5% carbon dioxide; to minimize changes in the isotopic composition of the nutrient solutions the gas was always bubbled through water of the same deuterium content as the nutrient being used. The growth medium employed for *Chlorella* originally had the composition (g/l): KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4$, 0.125; $FeSO_4 \cdot 7H_2O$, 0.0054. For *Scenedesmus*, a somewhat different nutrient solution was employed: (g/l) $MgSO_4$, 0.24; NH_4NO_3, 0.20; Na_2HPO_4, 0.10; $KHCO_3$, 0.10; $Ca(NO_3)_2 \cdot 4H_2O$, 0.22; NaCl, 0.01; $FeSO_4 \cdot 7H_2O$, 0.014. Both solutions had present as minor elements: (p.p.m.) B, 0.5; Mn, 0.25; Zn, 0.05; Cu, 0.02; Mo, 0.05. Although these solutions are adequate for growth, more recently we have employed for *Scenedesmus* a nutrient medium of double the concentration given above; growth is thereby significantly accelerated. We have also observed that the replacement of part of the potassium by sodium has a markedly beneficial effect on the growth of *Chlorella*.

The heavy water used in this work was supplied as 99.6% D_2O, and was purified by distillation from alkaline permanganate in a Barnstead conductivity-water apparatus. The tritium content of the deuterium was less than 0.01 μC/g. The purification of the heavy water is in our opinion a matter of some importance, since many early observations and perhaps even some recent ones may have been vitiated through the use of heavy water containing metal ion and other contaminants. It should be noted that we have used hydrogen-containing salts to prepare the nutrient solutions in D_2O. The amounts of hydrogen so introduced do not appreciably affect the hydrogen content of the 99.6% D_2O; of course this would not be true if D_2O of higher isotopic parity were employed.

Various cultures of algae were employed. Cultures of *Chlorella vulgaris* and *Chlorella pyrenoidosa* were kindly given us by the late Professor R. EMERSON. *Scenedesmus obliquus* was provided through the courtesy of Professor H. GAFFRON, and we have also obtained cultures of this organism from the algae culture collection of Indiana University.

New cultures were started by inoculation from cultures growing in an ordinary water medium or from ordinary agar slants. As deuterated cultures were obtained, they were used to inoculate other media of the same or higher deuterium concentration. The algae were not grown under sterile conditions.

Growth was followed by measurements of dry wt. Periodic microscopic examinations were made to observe morphological changes, cells in division, and the increase in the number of cells. At the end of the culture period the organisms were harvested by centrifugation.

Deuterium analyses

The deuterium content of the various media, of the intracellular water, and of the water from the combustion of dried algae was determined spectrophotometrically in microcells constructed for use with a Beckman DK recording spectrophotometer. The spectrophotometer cells were machined from 2.22-cm Teflon rod, provided with 2 filling holes (0.88 mm in diameter), and fitted with 9.5·1 mm quartz windows. The volume of sample required is approx. 20 μl; the optical path is 0.20 cm. Concentrations of deuterium were estimated from the absorption peak in the near infra-red at 1.66 μ. The cells were calibrated with known mixtures. The useful range of these cells is from 60 to 100 % deuterium. In the range from 10 to 60 % deuterium similar cells with an optical path of 0.088 mm may be used and concentrations estimated from the difference in optical density between absorption peaks at 1.44 and 1.66 μ. Details of the analytical method will appear elsewhere.

Intracellular algae water was obtained from packed cells by distillation under vacuum in small h-shaped vessels. The algae were then dried at 105–108° in vacuum for 24 h, and burned by a combustion procedure similar to that used by SMITH, TRACE AND BARBOUR[7]. The water of combustion was collected in a U-tube immersed in carbon dioxide snow. Carbon, hydrogen, nitrogen, and ash were determined by the Clark Microanalytical Laboratory, Urbana, Illinois and by Mr. W. SASCHEK of the University of Chicago. Freeze-dried algae are hygroscopic and must be preserved from contact with ordinary air to prevent dilution of deuterium by exchange.

Carbon dioxide fixation

Three flasks of *Scenedesmus* were grown for 3 weeks in a 99.6 % deuterated nutrient solution. The cultures were combined, harvested by centrifugation and suspended in fresh 99.6 % D_2O nutrient solution. An aliquot of this suspension was taken to determine the dry wt. of the cells, and the remainder was divided into 3 equal 100-ml vol., each containing 333 mg (dry wt.) of cells. Three determinations of carbon fixation were made with $^{14}CO_2$ as a tracer. Photosynthetic periods of 1 h and 5 h, and a dark period of 16 h were used. For each determination, the 100-ml vol. of algae suspension was placed in a 250-ml culture flask that was connected to a closed system consisting of a reservoir of $^{14}CO_2$ in air, an ionization chamber for continuous measurement of ^{14}C, and a pump for circulating the gas and for agitating the cells. The photosynthesis runs were carried out at 1500-ft candles of fluorescent light. All 3 experiments were done at 23°.

At the end of each fixation period the cell suspension was fixed by pouring into 500 ml of hot ethanol and boiling for 5 min. The cells were filtered through a small Soxhlet thimble and the extraction of the alcohol-soluble fraction continued in a Soxhlet apparatus for 8 h using 70 % aqueous ethanol. The ethanol extraction was followed by a 12-h extraction with water. An aliquot of each solution was plated out in an aluminum planchet and the ^{14}C activity determined by counting with a thin-window Geiger tube. The insoluble material was dried at 80° and the ^{14}C activity determined by combustion.

Control experiments using algae grown in ordinary nutrient solution were conducted in a similar manner, except that the algae were grown for only 10 days before exposure to $^{14}CO_2$, and each culture contained 182 mg (dry wt.) of cells.

RESULTS

Growth

Figs. 1 and 2 show growth curves for *Chlorella vulgaris* and *Scenedesmus obliquus* respectively, in media of various deuterium concentrations. All thesecul tures were begun by inoculation with algae grown in a non-deuterated medium. The algae in the 70 % deuterium medium made a greater gain in weight than the algae in the ordinary medium. In the 85 % deuterium medium *Chlorella vulgaris* grew essentially as well as in the ordinary medium. The growth of *Scenedesmus*, however, was delayed for 100 h at this concentration of deuterium, after which it proceeded at a normal rate. In the 99.6 % deuterium media, the growth of both *Chlorella* and *Scenedesmus* was inhibited for 200 h. After this initial period with no weight gain, growth began but proceeded at a much lower rate than that of the algae in ordinary media.

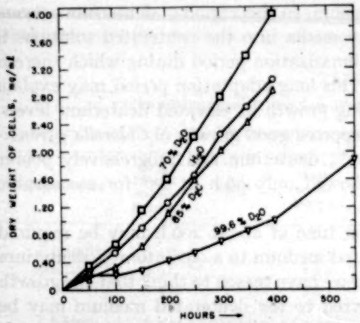

Fig. 1. Growth rates of *C. vulgaris* in solutions of varying solvent water composition. The solvent composition is indicated along the corsponding curve.

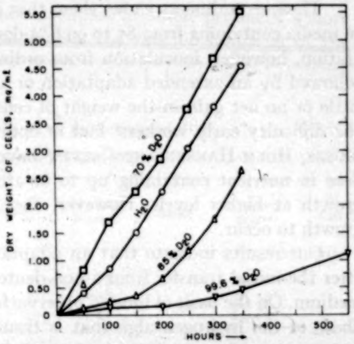

Fig. 2. Growth rates of *S. obliquus* in solutions of varying solvent water composition. The solvent composition is indicated along the corresponding curve.

These data indicate that at extremely high deuterium levels there is an extended period of adaptation or acclimatization before growth begins. This point is clearly illustrated in Fig. 3, which shows growth curves for *Scenedesmus* inoculated from an ordinary water medium into a 99.6 % deuterium medium and from a 99.6 atom % deuterium medium into a second such medium. The *Scenedesmus* taken from 99.6 % deuterium to 99.6 % deuterium showed immediate growth, while that taken from ordinary water to 99.6 % deuterium showed an inhibition period of about 200 h.

Similarly, when algae grown in 85 % deuterium or 99.6 % deuterium media are transferred back to an ordinary water medium, their growth rates are markedly retarded for 60 to 70 h. After this period they again grow at a normal rate.

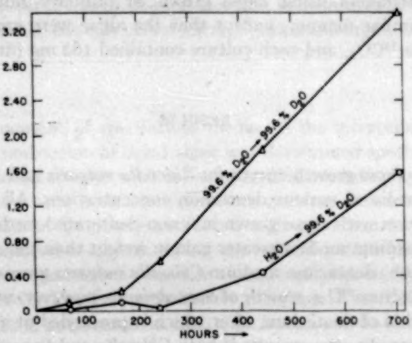

Fig. 3. Growth rates of *S. obliquus* in 99.6 % D_2O after transfer from a 99.6 % D_2O medium and after transfer from an ordinary H_2O medium. The growth of the algae transferred from the H_2O medium was inhibited for over 200 h.

These experiments show, then, that it is possible to obtain good growth of algae in media containing from 85 to 99.6 % deuterium. In this region of deuterium concentration, however, inoculation from ordinary media into the deuterated solutions is followed by an extended adaptation or acclimatization period during which there is little or no net gain in the weight of cells. This long adaptation period may explain the difficulty early workers[8] had in obtaining growth at elevated deuterium levels. Moses, Holm-Hansen and Calvin[4] have reported good growth of *Chlorella pyrenoidosa* in nutrient containing up to 60 atom % deuterium and progressively poorer growth at higher levels. However, they allowed only 96 h at 25° for measurable growth to occur.

Our results indicate that an adaptation time of about 200 h may be required after the initial transfer from a non-deuterated medium to a 99.6 atom % deuterium medium. On the basis of limited observations we have reason to think that the growth phase of the hydrogen alga that is transferred to the deuterated medium may be important in determining whether or not adaptation takes place and, if there is adaptation, the length of the inhibition period. Inhibition periods of 3 ½ and 5 ½, weeks were observed for a culture of *Chlorella pyrenoidosa*. In one case *Scenedesmus* failed to adapt after 18 weeks. Once growth has begun in the fully-deuterated mediume however, subsequent transfers to similar media produce immediate growth. Cultures in D_2O have now been maintained for more than 2 years. A number of changes in the deuterated media have improved growth rates so that, under our experimental conditions, we have cultures of *Chlorella* and *Scenedesmus* growing about 50 % as fast as those in ordinary water under our culture conditions.

Morphological changes

Morphological changes can be noted in algae freshly adapted to D_2O. Normally the *Scenedesmus* cell is an ellipsoid, but when it is first grown in high concentrations of D_2O, forms ranging from ellipsoids to spheres are found. *Chlorella vulgaris* cultures also show initially cells many times larger than normal when they are first grown in D_2O. Pigmentation is extremely granular, rather than diffuse as is normally the case. However, *Scenedesmus* grown for more than a year in 99.6 % D_2O are quite similar in appearance under the microscope to ordinary *Scenedesmus*; there is a suggestion that the deuterated *Scenedesmus* may have a rudimentary flagellum, but it is difficult to be certain. The most striking thing about the appearance of *Chlorella* grown for a year in 99.6 % D_2O is the noticeably larger size of the organism. In other respects the culture appears quite normal. It is of course possible that morphological changes reflecting the change in isotopic composition of the organisms do exist, and electron microscope and cytological studies are in hand.

Carbon dioxide fixation

Table I shows the distribution of fixed ^{14}C among the various fractions of *Scenedesmus*. In comparing the results of the 1-h light period to the 5-h light period, it is seen that the algae in the deuterated medium fixed considerably less carbon during the 5-h light period than do algae in ordinary water during a 1-h light period. This difference is especially marked in the carbon contained in the insoluble fractions. Fixed carbon seems to have accumulated in the alcohol-soluble fraction at the expense of the insoluble fraction. One may infer from these data that the rate of conversion of the early products of photosynthesis into more complex insoluble products is more strongly affected in 99.6 % deuterium media than is the rate of synthesis of the precursors.

TABLE I

DISTRIBUTION OF ^{14}C IN *Scenedesmus* AFTER EXPOSURE TO $^{14}CO_2$

All figures are expressed in $\mu C/g$ algae, dry.

% D_2O	*Exposure period*	*70% Ethanol soluble fraction*	*Ratio**	*Water soluble fraction*	*Ratio**	*Insoluble fraction*	*Ratio**	*Total ^{14}C fixed***	*Ratio**
0	1-h light	8.42		0.95		0.63		10.00	
0	5-h light	9.91		5.18		30.28		45.37	
0	16-h dark	5.45		2.97		4.41		12.83	
99.6	1-h light	3.52	0.42	0.30	0.32	0.35	0.56	4.17	0.42
99.6	5-h light	2.49	0.25	0.38	0.073	1.41	0.047	4.28	0.093
99.6	16-h dark	2.44	0.45	1.37	0.46	1.21	0.27	5.02	0.39

* Ratio of fixed carbon in the deuterated algae to fixed carbon in the control algae.
** Obtained by summation.

It is also interesting to note that during the 1-h light period and the 16-h dark period the deuterated algae fixed carbon approximately 0.40 as fast as the non-deuterated algae. Similar rate data were reported by CRAIG AND TRELEASE[9] on the rate of oxygen formation by non-deuterated algae suspended in an ordinary medium and in 99.9 % deuterium medium. They found that oxygen was evolved from the deuterated suspension 0.40 as fast as from the non-deuterated suspension.

Chemical and isotopic composition

Deuterium analyses showed (Table II) that in algae grown in deuterated media the intracellular water is essentially of the same composition as the extracellular water. Analyses of the water obtained from combustion of dry cells showed no detectable fractionation. We have not observed a preferential uptake of deuterium of the sort reported by MOSES, HOLM-HANSEN AND CALVIN[4]. After continued cultivation of *Scenedesmus*, thoroughly dried cells yielded water of combustion containing essentially 99 atom % deuterium.

TABLE II

DEUTERIUM OXIDE CONTENT OF VARIOUS ALGAE CULTURE FRACTIONS

Alga	*Per cent deuterium oxide*		
	Nutrient medium	*Cell water*	*Dry cells**
C. pyrenoidosa	68.6	69.5	67.4
C. vulgaris	69.8	70.8	68.8
C. vulgaris	—	99.5	98.1
C. vulgaris	99.7	99.7	98.2
S. obliquus	99.6	99.7	98.8

* Water of combustion.

The chemical composition of highly deuterated algae shows some variations when compared with the composition of non-deuterated algae (Table III). Both deuterated species show a small drop in carbon content as compared to the non-deuterated cells. The deuterated algae tend to have a somewhat elevated nitrogen content. On the whole, however, deuterated organisms show no pronounced differences in gross composition, although important changes in chemical composition are by no means excluded.

TABLE III

CHEMICAL COMPOSITION OF ALGAE

Algae	*Per cent D_2O*	*Per cent C**	*Per cent H**	*Per cent N**	*Total C, H, N*	*Per cent Ash*
S. obliquus	0	54.8	8.25	2.09	65.1	3
S. obliquus	0	53.6	6.97	4.98	65.6	2.7
S. obliquus	70	50.8	6.56	3.50	60.9	7
S. obliquus	85	51.1	7.10	7.29	65.6	7
S. obliquus	99.6**	52.0	6.79	4.82	63.6	4.8
S. obliquus	99.6**	49.4	6.78	3.45	59.6	2.8
C. vulgaris	0	57.6	6.96	4.76	70.3	4
C. vulgaris	70	54.1	7.78	3.97	65.8	6
C. vulgaris	85	51.1	7.62	5.36	65.1	5
C. vulgaris	99.6**	49.6	6.67	6.56	62.8	3.6

* Calculated on an ash-free, deuterium-free basis.
** After many transfers through fully deuterated media.

Isolation of deuterated sugars

14 g of dry, deuterated *Scenedesmus* cells were exhaustively extracted with 80 % ethanol and then with methanol[10]. The extracted cells were then hydrolyzed in 1 *M* DCl. The resultant solution was purified by addition of silver carbonate and passage

through a deionizing column. The clear aqueous solution was then evaporated to a small volume and fractionated chromatographically on a cellulose column.

As indicated by paper chromatographic analysis, a number of essentially pure components were isolated. The main components appeared to be glucose and mannose. The glucose fraction from the cellulose column was decolorized and deionized, brought almost to dryness, and triturated with absolute alcohol. Approx. 1.5 g of dry, white powder was obtained. Combustion of a sample of this material yielded water containing 56.8% deuterium, indicating about 97% deuteration at the non-exchangeable positions of glucose. The melting point of the powder was in the range of 145.0–146.5°. After recrystallizing, the melting point was 145.5–147.0°. Authentic glucose melted at 146.0–147.0°, and a mixed melting point showed no depression.

DISCUSSION

The experiments described here indicate that algae do indeed grow and divide in a medium containing more than 99% D_2O. After an inhibition period both *Chlorella* and *Scenedesmus* grew and divided, and after growth was established showed only a small percentage of abnormally large cells. We believe that it will be possible to formulate improved nutrient media for deuterated organisms since it is unlikely that a nutrient which is optimum for H_2O will necessarily be optimum for D_2O. We also see no *a priori* reasons why other autotrophic organisms should not be available to culture in highly deuterated environments.

Deuterated compounds other than sugars can be isolated from algae. Further, it may be possible to use deuterated algae as such as a substrate for the growth or organisms that would give high yields of specific compounds such as amino acids, nucleic acids, and antibiotics. Simultaneous labeling with ^{14}C can also easily be accomplished.

With the successful culture of algae in deuterated media the way is open for a considerable variety of experiments involving deuterium and its biological effects. Practical application may reasonably be expected to be forthcoming.

ACKNOWLEDGEMENTS

We are deeply indebted to Dr. L. KAPLAN for measuring the tritium content of the deuterium oxide, and for ^{14}C measurements.

This investigation was based on work performed under the auspices of the U.S. Atomic Energy Commission.

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TRIVALENT CATION BINDING OF ACID MUCOPOLYSACCHARIDES

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(Received April 1st, 1959)

SUMMARY

The "outer sphere" association of $Co(NH_3)_6^{+3}$ with acid mucopolysaccharides of connective tissue has been investigated by a spectrophotometric procedure. The data fit a linear form of the LANGMUIR adsorption isotherm and allow calculation of parameters characterizing the binding affinity and the number of binding sites. A similar equation, based upon competition between trivalent cation and Na^+ for binding sites, was derived and accounts approximately for the dependence of association on Na^+ concentration. The method distinguishes specific site-binding, or ion-pair formation, from non-localized retention of microions in the potential field of the macroion. It is suggested that the binding affinities depend largely on electrostatic interactions between neighboring charged groups on the polyion. In this manner, the high affinities of heparin preparations may be accounted for, and differences between isomeric chondroitin sulfates related to structural differences between the respective repeating disaccharide periods.

INTRODUCTION

Binding of cations, including $Co(NH_3)_6^{+3}$, to acid mucopolysaccharides such as chondroitin sulfate from cartilage, has been extensively investigated since 1931 by BUNGENBERG DE JONG and collaborators[1]. The methods used, however, do not lead readily to a quantitative interpretation of the binding.

The observation by LINHARD[2] that anions can cause marked changes in the absorption spectra of Co(III) has led several investigators[3–6] to utilize spectral methods for the determination of equilibrium constants for the association of $Co(NH_3)_6^{+3}$ and divalent anions. Since the inner sphere of the ion $Co(NH_3)_6^{+3}$ remains intact, the term "outer sphere association" has been applied[5] to indicate the type of complex formed. It was the purpose of the work reported here to obtain, if possible, constants characterizing outer sphere association of $Co(NH_3)_6^{+3}$ with high molecular polyanions such as acid mucopolysaccharides.

MATERIALS AND METHODS

Three isomeric acid mucopolysaccharides of connective tissue (see review by MEYER *et al.*[7]) with disaccharide repeating units containing equimolar amounts of hexuronic

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* Work done during tenure of an Established Investigatorship of the American Heart Association.

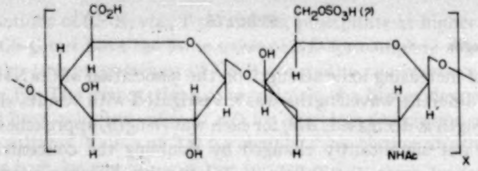

Fig. 1. Disaccharide repeating period of isomeric chondroitin sulfates A and C. Chondroitin sulfate B contains L-iduronic acid, the C-5 epimer of D-glucuronic acid shown. The position of the sulfate group is uncertain, but probably is in the 6-position shown for C and in the 4-position for A and B.

acid, N-acetyl-D-galactosamine, and ester sulfate were employed. Current knowledge of the structures of these repeating units is summarized in Fig. 1. Chondroitin sulfate A (CS-A) was obtained from bovine nasal cartilage[8]; chondroitin sulfate B (CS-B), derived from beef lung[9], was a gift from Hoffmann-LaRoche & Co., Basle, courtesy of Dr. A. WINTERSTEIN; chondroitin sulfate C (CS-C) derived from a chordoma[7], was a gift from Dr. K. MEYER. The properties of these polysaccharides have been further described in earlier publications[9, 10–12]. Sodium hyaluronate, from Group A streptococcus, was a purified preparation, courtesy of Dr. J. A. CIFONELLI. Heparin I, obtained from Abbott Laboratories, North Chicago, Illinois, showed on analysis an average of two sulfate groups per disaccharide repeating unit. Heparin II, obtained from Hoffmann-LaRoche & Co., courtesy of Dr. A. WINTERSTEIN, showed on analysis an average of three sulfate groups per disaccharide repeating unit. All preparations were the sodium salts with the anhydrous content based upon chemical analyses for N, hexosamine, uronic acid, and sulfate, or, in the case of the chondroitin sulfates, based upon refractive increment[11].

$Co(NH_3)_6Cl_3$, prepared according to BJERRUM AND MCREYNOLDS[13], was converted to the perchlorate salt, and purified by several recrystallizations. $HClO_4$ and $NaClO_4$ were of A. R. quality. Perchlorate ion is used[6] since, unlike chloride ion, it has a negligible effect on the extinction of $Co(NH_3)_6^{+3}$.

Spectral measurements were made with a Beckman Model DU Quartz spectrophotometer fitted with a cell compartment, the temperature of which could be controlled by circulating fluid at 25° ± 0.1. The optical density, $D = \log (I_0/I)$, was measured for 1-cm cells against a blank from which polysaccharide and $Co(NH_3)_6^{+3}$ were omitted. D is the O.D. of a solution containing polysaccharide and $Co(NH_3)_6^{+3}$, corrected for a small absorption due to the polysaccharide alone, and D_0 is the O.D. of a solution of $Co(NH_3)_6^{+3}$ at the same concentrations. The increment in O.D. due to association is thus $\Delta D = D - D_0$.

The range of concentrations that could be employed was limited by precipitation of the cobaltammine salt of the polysaccharide at low ionic strengths. Such precipitation could be detected by a Tyndall effect or by a time dependent light absorption. D_1, the O.D. of a solution in which all the $Co(NH_3)_6^{+3}$ is associated with polyion, was determined at the lowest ionic strength for which clear solutions existed. An increase in concentration of polyanion then produced no significant increase in O.D. due to association. If the free cation and the cation–polyanion complex have extinction coefficients independent of the concentrations of the components of a solution, then the fraction of cation associated to polyanion in a solution is given by

$$f = \Delta D/(D_1 - D_0) \qquad (1)$$

RESULTS

Effect of wavelength

The effect of increasing ionic strength on the association of $Co(NH_3)_6^{+3}$ and CS-A (prep. CS-8) at different wavelengths was investigated with results shown in Fig. 2. As the ionic strength is decreased, ΔD, for each wavelength, approaches an asymptotic value, which is not significantly changed by doubling the concentration of CS-A. Within experimental error, the values of ΔD for the different wavelengths at the same ionic strength are in constant ratio. Thus, the degree of association determined in the spectral measurements is independent of the wavelength used.

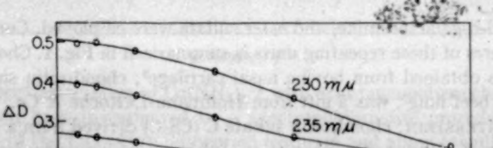

Fig. 2. Cation association at different wavelengths. Concentrations were $1.00 \cdot 10^{-3}$ *M* for $Co(NH_3)_6(ClO_4)_3$ and $2.87 \cdot 10^{-3}$ *M* in repeating period for CS-A (prep. CS-8).

Effect of ionic strength

The effect of increasing ionic strength on the association of complex cation with acid mucopolysaccharides is illustrated in Fig. 3. Results similar to those shown for CS-8 were obtained with preparations CS13F and CS3E2A, previously described[11]. Association to CS-A is thus independent of molecular weight in the range 17,000 to 50,000. The curve for CS-C ($M_w = 40{,}000$) is likewise superimposable on the one for CS-8.

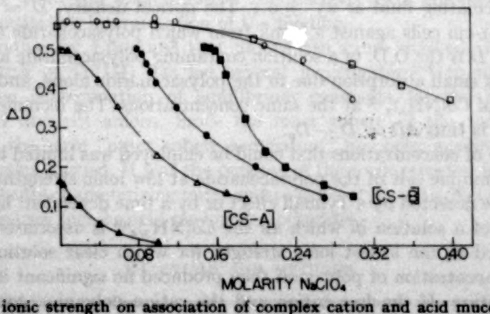

Fig. 3. Effect of ionic strength on association of complex cation and acid mucopolysaccharide. Concentrations are $1.00 \cdot 10^{-3}$ *M* in $Co(NH_3)_6(ClO_4)_3$ and $2.87 \cdot 10^{-3}$ *M* in repeating period for each polysaccharide. The wavelength is 230 mμ, except for heparin I and heparin II which is 240 mμ. Data points are given for sodium hyaluronate (▲), CS-8 (△), F3A (■), F6 (●), heparin I (○), and heparin II (□).

The two fractions of CS-B, viz., F3A and F6, precipitate at higher ionic strengths than CS-A and CS-C but have the same value of ΔD for complete association. Differences between the two preparations of CS-B are due to the presence of about 20% CS-A in fraction F6. The preparations of heparin show a higher degree of association with cation and have a higher value of ΔD for complete association at 240 mμ than the chondroitin sulfates. Values of ΔD for complete association at other wavelengths are estimated as 0.78 for 235 mμ and 1.00 for 230 mμ.

Sodium hyaluronate has a comparatively low affinity for the complex cation. Also, the value of ΔD in the absence of $NaClO_4$ may be increased by 40% upon doubling the concentration of polysaccharide. Thus complete association of complex cation was not attained for this polysaccharide.

Association at constant ionic strength

Further association data were obtained at a fixed concentration of $NaClO_4$, which serves to maintain an essentially constant ionic strength, a concentration of $2.87 \cdot 10^{-3}$ *M* in repeating period for each polysaccharide, and a variable concentration of $Co(NH_3)_6(ClO_4)_3$. The fraction of complex cation associated was calculated from eqn. (1).

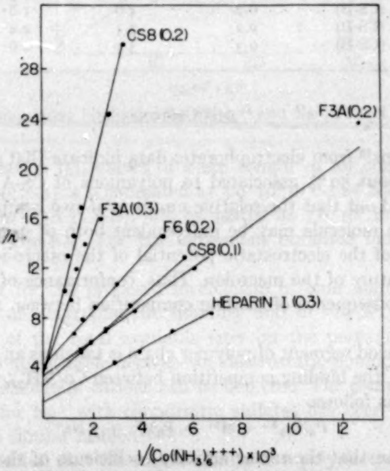

Fig. 4. Association of $Co(NH_3)_6^{+3}$ and acid mucopolysaccharides at constant concentration of $NaClO_4$. The polyanion concentration in all cases is $2.87 \cdot 10^{-3}$ *M* in repeating period. Molarity of $NaClO_4$ is indicated for each curve.

The data, plotted in Fig. 4, fit a linear form of the LANGMUIR[14] adsorption isotherm, a consequence of the law of mass action

$$\frac{1}{r} = \frac{1}{n\,k}\,\frac{1}{(\text{cation})} + \frac{1}{n} \qquad (2)$$

where $r = \dfrac{\text{moles bound cation}}{\text{total repeating periods}}$

and n = binding sites per repeating period

This expression is analogous to one derived by KLOTZ[15] for small ion binding to a protein molecule which has a fixed number n of independent binding sites, each with the same intrinsic affinity constant k for the small ion.

Parameters of eqn. (2) for each polysaccharide are shown in Table I. The data for heparin indicate the presence of one binding site per disaccharide repeating unit of each polysaccharide molecule. For the chondroitin sulfates the data indicate apparently one binding site for three repeating periods. No appreciable change in parameters occurs in experiments in which the polysaccharide concentration is lowered by 50 %.

TABLE I

PARAMETERS OF ASSOCIATION OF $Co(NH_3)_6^{+3}$ AND ACID MUCOPOLYSACCHARIDES AT 25°

Preparation	*NaClO₄ molarity*	$1/n\ \frac{\text{(repeating period)}}{\text{(binding site)}}$	k
Heparin II	0.5	1.1	$1.0 \cdot 10^3$
Heparin I	0.3	1.2	$1.1 \cdot 10^3$
CS-8 (CS-A)	0.2	3.4	$0.4 \cdot 10^3$
CS-8 (CS-A)	0.1	2.7	$1.5 \cdot 10^3$
F6 (CS-B)	0.2	4.0	$1.8 \cdot 10^3$
F3A (CS-B)	0.2	3.4	$2.4 \cdot 10^3$
F3A (CS-B)	0.3	3.4	$0.6 \cdot 10^3$

DISCUSSION

Previous calculations[12] from electrophoretic data indicate that at 0.1 ionic strength, sodium ions are about 50 % associated to polyanions of CS-A and CS-B. LIFSON[16] has recently pointed out that the relative numbers of two competing ions associated to a polyelectrolyte molecule may be independent both of nearest neighbour interaction energy and of the electrostatic potential of the macro-ion and consequently of the polymeric nature of the macroion. Thus, conformance of the data to eqn. (2) may be largely a consequence of binding competition between trivalent cations and sodium ions.

If, then, a 3-period segment of polymer chain is taken as an independent binding unit, designated P_3, the binding competition between $Co(NH_3)_6^{+3}$ (written M^{3+}), and Na^+ is formulated as follows

$$P_3Na_3^{3-} + M^{3+} = P_3M^{3-} + 3\ Na^+ \tag{3}$$

With the assumption that the molar activity coefficients of the two polysaccharide ions are identical, the equilibrium expression is given by the following in which the ionic charges are omitted for convenience:

$$(P_3M) = \frac{K(P_3Na_3)\ y_M(M)}{y^3_{Na}\ (Na)^3}$$

Since $(P_3)_T = (P_3M) + (P_3Na_3)$, we obtain by substition

$$\frac{(P_3)_T}{(P_3M)} = \frac{y^3_{Na}\ (Na)^3}{Ky_M(M)} + 1 \tag{4}$$

Eqn. (2) is in the same form as the above equation when both sides of eqn. (2) are multiplied by $n = 1/3$, when $k = \frac{K\ y_M}{y^3_{Na}(Na)^3}$ (5)

Since $(\mathrm{M})_T = (\mathrm{M}) + (P_3\mathrm{M})$, substituting in eqn. (4) and rearranging terms yields

$$F(P_3\mathrm{M}) = \frac{(P_3)_T - (P_3\mathrm{M})}{(P_3\mathrm{M})} - (P_3)_T - (\mathrm{M})_T + (P_3\mathrm{M}) = \frac{y^3_{\mathrm{Na}}(\mathrm{Na})^3}{K\, y_{\mathrm{M}}} \tag{6}$$

The left hand side of eqn. (6) is plotted against the stoichiometric Na^+ concentration in Fig. 5. Absence of a constant slope over the entire concentration range is not surprising in view of expected variations of both activity coefficients as well as of the electrostatic potential of the macroion. Values of $K\, y_{\mathrm{M}}/y^3_{\mathrm{Na}}$ at high Na^+ concentrations are estimated as ~3.0 and ~13, respectively, for CS-8 and F3A.

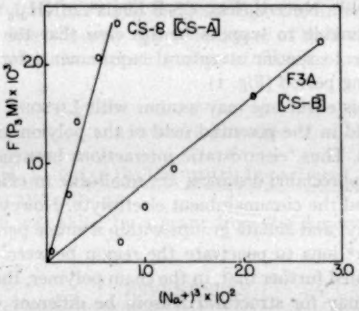

Fig. 5. Competitive binding of $\mathrm{Co(NH_3)_6^{+3}}$ and $\mathrm{Na^+}$. Plot of eqn. (6).

By means of relation (5), values of k are computed for comparison with those of Table I. For CS-8 (CS-A), k is estimated as $3.0 \cdot 10^3$ in 0.1 M $\mathrm{NaClO_4}$ and as $0.4 \cdot 10^3$ at 0.2 M $\mathrm{NaClO_4}$. For F3A (CS-B), k is estimated as $1.6 \cdot 10^3$ in 0.2 M $\mathrm{NaClO_4}$ and as $0.5 \cdot 10^3$ in 0.3 M $\mathrm{NaClO_4}$. The fair agreement obtained indicates the essential validity of eqn. (3).

Further support for the proposed mechanism of ionic competition is obtained from measurements of electrophoretic mobility and of viscosity. Under conditions where 50% or less of the total available sites on the polyanion are occupied by trivalent cations, no appreciable decrease in electrostatic potential of the polyion as a consequence of exchange of cations can be detected. The comparable phenomenon of exchange of $\mathrm{H^+}$ for $\mathrm{Na^+}$ with chondroitin sulfates has been previously observed and interpreted in a similar fashion[10, 12].

It might be expected that the cation affinity of a polyelectrolyte molecule would depend upon the average electrostatic potential of the macroion. Indeed, the order of cation affinity (Fig. 3), hyaluronate < chondroitinsulfate < heparin I < heparin II, is that of increasing linear charge density of these polysaccharides. Also, ΔD for the chondroitin sulfates is decreased as much as 90% upon lowering the pH to 2.0 with $\mathrm{HClO_4}$, an action which discharges the carboxyl groups. Thus, contrary to the view of VOURAS AND SCHUBERT[17], the ionized carboxyl groups, as well as the sulfate groups, participate in association of cations.

In order to account more fully for the observed behavior of some polyelectrolytes, OSAWA, IMAI AND KAGAWA[18] have assumed that neighboring charges on a polyanion may interact electrostatically with sufficient strength so that small univalent cations may be bound to negative sites (P-bound). These cations are to be distinguished from others retained in a non-localized manner in the potential field of the macroion (ψ bound). This concept of binding has been further advanced by HARRIS AND RICE[19] who assume that the binding follows a mass action law. Electrostatic interaction of neighboring acidic groups might thus be of major importance in accounting for the high binding affinities for cations of the polysaccharides studied, and for the observed differences between the isomeric chondroitin sulfates.

Chondroitin sulfates A and B are linear polyelectrolytes with identical polymer chain characteristics in dilute aqueous solutions. Their electrophoretic surface potentials are the same[13]. Nevertheless, CS-B binds $Co(NH_3)_6^{+3}$ much stronger than does CS-A. It is reasonable to suppose in this case that the increased affinity for trivalent cation is due to specific structural requirements for increased binding at the site of the repeating period (Fig. 1).

To account for this effect one may assume with LIFSON[16] that most of the Na^+ ions associated are held in the potential field of the polyion and comparatively few bound to specific sites. Thus, electrostatic interactions between P_3 segments, which are separated by an appreciable distance, are small due to efficient shielding by the associated Na^+ ions and the circumambient electrolyte. However, electrostatic interaction between carboxyl and sulfate groups within a single period, may be large due to the inability of Na^+ ions to penetrate the region between these relatively close groups. It should be noted further that, in the chain polymer, the degree of interaction of a carboxyl group may for structural reasons be different with each of the two adjacent sulfate groups. The finding that CS-B binds H^+, as well as $Co(NH_3)_6^{+3}$, stronger than does CS-A[12], is consistent with the hypothesis that the increased affinity of CS-B over CS-A for site-bound cations is due to the greater electrostatic interactions between adjacent carboxyl and sulfate groups in the CS-B molecule.

POSEY AND TAUBE[6] obtained values of the molar extinction coefficient at 24.5° for $[Co(NH_3)^{+3}\,SO_4^{=}]^+$ of 788 at 235 mμ and 388 at 240 mμ. They suggested that an apparent increase in ε at high $SO_4^{=}$ concentration was due to a second stage of outer sphere association yielding the ion $[Co(NH_3)_6^{+3}\,(SO_4^{=})_2]^-$. Corresponding values for the chondroitin sulfates of $\varepsilon_{235} = 810$, $\varepsilon_{240} = 400$ and for heparin of $\varepsilon_{235} = 1180$, $\varepsilon_{240} = 680$ suggest that the cation may be bound to two singly charged anionic groups in the chondroitin sulfates and to three anionic groups (one period) in heparin. The

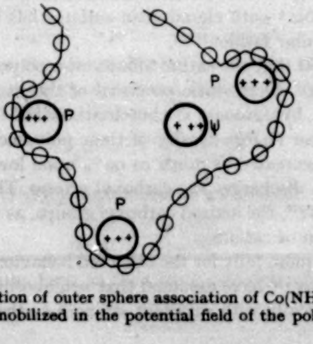

Fig. 6. Schematic representation of outer sphere association of $Co(NH_3)_6^{+3}$ to chondroitin sulfate as ion-pairs (P). Cations immobilized in the potential field of the polyanion are indicated by ψ.

postulated binding to chondroitin sulfates is illustrated in Fig. 6. Two nearest neighbor periods, in addition to the single period site of cation association are excluded from simultaneous occupation by any other trivalent cation. Since the occupied periods are otherwise not distinguishable (except for the two periods at the ends of the chain) from the excluded periods, the two types of periods are interchangeable. The independence of *k* from polymer concentration indicates that appreciable association of a single cation to anionic groups of different chains, although present in related systems[20], did not occur in these experiments.

The order of cation affinities of the sulfated acid mucopolysaccharides is that of their anticoagulant activities[9]. This suggests that the anticoagulant properties of these substances may depend partly upon the degree of their electrostatic interaction with small cations or with the cationic groups of proteins involved in blood coagulation.

ACKNOWLEDGEMENTS

I am indebted to Dr. H. TAUBE of the Chemistry Department, University of Chicago, for helpful suggestions and criticism.

This investigation was aided by a grant from the American Heart Association.

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CARBOHYDRATE METABOLISM OF MAMMARY TISSUE

III. FACTORS IN THE REGULATION OF PATHWAYS OF GLUCOSE CATABOLISM IN THE MAMMARY GLAND OF THE RAT

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(Received April 8th, 1959)

SUMMARY

1. The effect of insulin in stimulating the formation of $^{14}CO_2$ from [1-^{14}C]glucose by mammary gland slices has been further investigated.

2. No changes were found in the activities of hexokinase, G-6-P or 6-PG dehydrogenases, TPNH-cytochrome *c* reductase or TPNH-glutathione reductas ein the mammary gland following insulin treatment.

3. Insulin has a much smaller effect on the oxidation of carbon-1 of glucose and the incorporation of glucose carbon into fatty acids by mammary tissue from pregnant rats than on mammary tissue from lactating rats.

4. Phenazine methosulphate, pyocyanin and methylene blue all cause a preferential stimulation of the oxidation of carbon-1 of glucose; DNP and dicoumarol increase the oxidation of carbon-6 of glucose by mammary gland slices.

5. High glucose concentrations increased the oxidation of carbon-1 of glucose and the incorporation of glucose carbon into fatty acids; the oxidation of carbon-6 of glucose appeared to be independent of the glucose concentration.

6. The action of insulin and of redox systems on the metabolism of glucose have been discussed; it is suggested that the supply of oxidised TPN is one of the factors controlling the activity of the pentose phosphate pathway.

INTRODUCTION

There is considerable evidence that a large part of the glucose catabolism of the lactating rat mammary gland occurs by way of the pentose phosphate pathway[1, 2]. It has been calculated, on the basis of the incorporation of ^{14}C from specifically labelled glucoses into fatty acids, that of the glucose utilized approximately 50 % was metabolised by a non-glycolytic route[1]. ABRAHAM *et al.*[3] have shown that the addition of insulin *in vitro* to slices of lactating rat mammary tissue results in an

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Abbreviations: TPN$^+$ and TPNH, the oxidised and reduced forms of triphosphopyridine nucleotide respectively; G-6-P, D-glucose-6-phosphate, 6-PG, 6-phosphogluconate; F-6-P, fructose-6-phosphate; DNP, 2,4-dinitrophenol;

$$\text{C-1/C-6}, \frac{\text{Yield of } ^{14}CO_2 \text{ from [1-}^{14}\text{C]glucose}}{\text{Yield of } ^{14}CO_2 \text{ from [6-}^{14}\text{C]glucose}}.$$

approximately two-fold increase in the $^{14}CO_2$ production from [1-^{14}C]glucose whereas the $^{14}CO_2$ formed from [6-^{14}C]glucose is decreased somewhat. Insulin also has a pronounced stimulating effect *in vitro* on lipogenesis in lactating rat mammary gland slices (see FOLLEY AND MCNAUGHT[4]). These factors, the high activity of the pentose phosphate pathway and the striking *in vitro* effects of insulin, make the lactating mammary gland particularly suitable for studying the regulation of the two alternative pathways of glucose catabolism. The present experiments are, in part, concerned with the effect of insulin on the activity of some of the enzymes of the pentose phosphate pathway and of two of the enzymes involved in the reoxidation of TPNH.

There is growing evidence showing that the supply of TPN$^+$ is one of the factors controlling the activity of the pentose phosphate pathway in many tissues[5-8]. In the rat mammary gland, as in most animal tissues, this coenzyme is present almost exclusively in the reduced form[9,10]. A study has therefore been made of the effect of three artificial electron acceptors on the production of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose using tissue slices from rat mammary glands at different functional stages. In addition, the effect of glucose concentration and of the uncoupling agents DNP and dicoumarol on the formation of $^{14}CO_2$ from specifically labelled glucoses and the incorporation of glucose carbon into fatty acids has been measured. A preliminary account of part of this work has been published[11].

EXPERIMENTAL

Animals

Adult female primiparous rats of approximately 200 g were used. Litters were in all cases reduced to 8 pups. Only the abdominal mammary glands were used. In the experiments reported in Table I rats at the mid-lactation period were anaesthetised with sodium amytal (10 mg/100 g body weight) and the left abdominal mammary gland was removed and used immediately for the preparation of tissue slices and homogenates. The anaesthetised rat was then injected intravenously with 10 I.U. insulin and 1 h later the right abdominal mammary gland was removed and used for comparable experiments. In experiment 3, Table I, 18 mg of glucose were given in three divided doses during the hour between the injection of insulin and the removal of the second gland in order to counteract the effect of insulin on the blood sugar level.

The adipose tissue used in the experiments reported in Table IV was obtained from young male rats weighing approximately 200 g. The method of removal and of handling the epididymal fat closely followed that described by WINEGRAD AND RENOLD[12].

Materials

TPN, G-6-P, ATP and cytochrome *c* were commercial products of the Sigma Chemical Co., St. Louis, Mo. 6-PG and 2-deoxyglucose were preparations used previously[13]. Oxidised glutathione was prepared from glutathione (donated by Distillers Co. Ltd.), by the method of PIRIE[14]. Phenazine methosulphate was prepared from phenazine (Light and Co.) by the method of HILLEMANN[15]. [1-^{14}C]glucose and [6-^{14}C]glucose were obtained from the Radiochemical Centre, Amersham. The crystalline insulin, free from hyperglycaemic-glycogenolytic factor, used in these experiments was kindly given by Dr. HALLAS-MØLLER, Novo Terapeutisk Laboratorium.

METHODS

Enzymes

Hexokinase, G-6-P dehydrogenase, 6-PG dehydrogenase, TPNH-cytochrome *c* reductase and phosphoglucose isomerase activities were estimated as described previously[10, 13] and expressed in units/g tissue corrected for the "milk error" as described by FOLLEY AND GREENBAUM[16]. The definition of a unit of activity for each of these enzymes is as follows:

Hexokinase. The quantity of enzyme which catalyses the phosphorylation of 1.0 μmole 2-deoxyglucose in 15 min at 30°.

G-6-P dehydrogenase and 6-PG dehydrogenase. The quantity of enzyme which reduces 1.0 μmole TPN/min at 20°.

TPNH-cytochrome *c* reductase. The quantity of enzyme which reduces 1.0 μmole cytochrome *c*/min at 20°.

Phosphoglucose isomerase. The quantity of enzyme which produces 1.0 μmole F-6-P/min at 37°.

Estimation of TPNH-glutathione reductase

The activity of this enzyme was measured by the method of RACKER[17]. Mammary gland homogenates, prepared in a Potter homogeniser using 9 volumes of ice cold 0.15 *M* KCl containing $1.6 \cdot 10^{-4}$ *M* $KHCO_3$, were centrifuged at 8000 × *g* for 15 min in an M.S.E. Angle 13 centrifuge. The supernatant was removed and dialysed against the same extracting medium at 2°. This fraction contained approximately 95 % of the total enzyme activity. The enzyme activities were determined spectrophotometrically by following the rate of reoxidation of TPNH (prepared by the method of KAPLAN *et al.*[18]) at 340 mμ in 1-cm cells using 0.1 or 0.2 ml of mammary gland supernatant fraction. A blank containing all the components of the assay system except oxidised glutathione was included. This blank value was found to be very low or negligible. A unit of enzyme activity is defined as the amount of enzyme oxidising 1.0 μmole TPNH/min at 20°. The intracellular distribution of this enzyme in the mammary gland is similar to that of rat liver[19].

Isotope experiments

500 mg of mammary gland slices or approximately 250 mg epididymal fat pad were incubated with 4.5 ml Krebs-Ringer bicarbonate solution and 20 mg of glucose containing 0.2 μC labelled glucose in the form of either [1-^{14}C]glucose or [6-^{14}C]-glucose. The gas phase was $O_2 + CO_2$ (95 : 5). After incubation for 90 min at 37°, 1 ml 5 *N* KOH was introduced into the centre well and 0.5 ml 5 *N* H_2SO_4 into the main chamber of the flask by injection through the rubber cap. The $^{14}CO_2$ content was determined after precipitation with $BaCl_2$ containing NH_4Cl, using the "infinitely thick" layer technique. The activity of the labelled glucose used in the reaction vessels was similarly determined after complete oxidation to $^{14}CO_2$ with potassium persulphate[20].

At the end of the incubation period the mammary gland tissue slices were removed, washed and refluxed with 3 % sodium ethylate for 2 h. The resulting solution was used for the determination of the specific activity of the fatty acids as described by GREENBAUM AND GLASCOCK[21]. Approximately 2 mg of the fatty acids, dissolved in petroleum ether (b.p. 60–80°), were plated on 2 cm^2 discs covered with lens tissue. The activity of these samples was measured in a flow counter (Tracerlab SC 16). These results are expressed as counts/min/mg fatty acid.

In the experiments reported in Table VI the specific activity of the added glucose was maintained at a constant value of 0.1 μC/10 mg glucose.

RESULTS

Effect of insulin in vivo *on the metabolism of glucose by the mammary gland*

The effect of insulin treatment on the incorporation of ^{14}C from specifically labelled glucoses into $^{14}CO_2$ and fatty acids by the lactating rat mammary gland is summarised in Table I. In each of these experiments there is a marked stimulation in the production of $^{14}CO_2$ from [1-^{14}C]glucose and an increase in the C-1/C-6 quotient. The incorporation of ^{14}C from [6-^{14}C]glucose into fatty acids was also greatly increased by insulin treatment. When the *in vivo* and *in vitro* effects of insulin were compared (Expt. 1 Table I) the results show that an essentially similar pattern of change occurred, the response in the presence of the high doses of insulin *in vitro* being somewhat greater. The *in vitro* effects of insulin found here were similar to those previously reported by ABRAHAM *et al.*[3].

The effect of insulin in causing such a marked increase in the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose was further investigated by measuring the activity of some of the enzymes concerned in the production of $^{14}CO_2$ *via* the pentose phosphate pathway, namely; hexokinase, G-6-P dehydrogenase and 6-PG dehydrogenase, in mammary tissue before and after insulin treatment. As shown in Table I, no changes occurred in the activity of any of these enzymes. Since the high activity of the enzymes of the pentose phosphate pathway may make the rate of reoxidation of TPNH a limiting reaction in mammary tissue[10], the effect of insulin on TPNH-cytochrome *c* reductase and TPNH-glutathione reductase activity of mammary tissue was also measured. The activities of these enzymes were not increased by treatment of the animal with insulin, in fact a small inhibition (approximately 20 %) was found in the activity of TPNH-glutathione reductase. This may be related to the zinc content of the insulin preparation used since LANGDON[22] has shown that the purified enzyme from liver is extremely sensitive to zinc ions.

The activity of TPNH-glutathione reductase is quite high in the lactating mammary gland, comparable with that of TPNH-cytochrome *c* reductase and about 5 times greater than that of the mitochondrial pyridine nucleotide transhydrogenase[10]. It is possible, therefore, that this enzyme plays an important role in mammary gland metabolism.

Insulin sensitivity of rat mammary tissue at different functional stages

The activity of the pentose phosphate pathway undergoes very striking alterations at different functional stages of the mammary gland[13]. It seemed of interest, therefore, to compare the effects of insulin on the oxidation of specifically labelled

TABLE I

EFFECT OF INSULIN *in vivo* ON GLUCOSE METABOLISM OF LACTATING RAT MAMMARY TISSUE

Dose of insulin*-(I.U.)	% conversion of labelled glucose to $^{14}CO_2$§		$^{14}CO_2$ from G-1-^{14}C / $^{14}CO_2$ from G-6-^{14}C	Specific activity§§ of fatty acids derived from		Units of enzyme/g tissue§§§					
						Hexokinase	G-6-P dehydrogenase	6-PG dehydrogenase		TPNH-cytochrome c reductase	TPNH-glutathione reductase
	[1-^{14}C]glucose	[6-^{14}C]glucose		G-1-^{14}C	G-6-^{14}C			pH 9.0	pH 7.6		
0	11.38	2.32	4.91	88	144	80	19.35	7.52	3.68	2.51	2.28
10	19.35	3.04	6.40	124	300	70	17.05	7.15	3.34	2.65	1.74
1/flask**	28.38	9.57	50.20	56	436						
0	8.94	1.34	6.40	66	134	78	20.80	6.30	3.15	3.15	1.62
10	12.36	0.79	15.70	86	374	77	20.10	6.43	3.19	2.74	1.30
0***	5.85	2.13	2.74	64	116	54	12.36	4.10	1.84	1.65	1.68
10	9.79	1.39	7.05	48	428	51	11.90	3.92	1.71	1.73	1.32

* Anaesthetised rat injected intravenously with 10 I.U. insulin after removal of left abdominal mammary gland, the right abdominal mammary gland removed 1 h later. Stage of lactation-11th day.

** 1 I.U. insulin added to incubation medium of control for comparison of *in vivo* and *in vitro* effects on glucose metabolism.

*** 18 mg glucose given in 3 divided doses during hour between injection of insulin and removal of second gland.

§ 500 mg mammary gland slices incubated for 1.5 h at 37° in 4.5 ml Krebs-Ringer bicarbonate solution with 20 mg glucose containing 0.2 μC ^{14}C in the form of [1-^{14}C]glucose or [6-^{14}C]glucose. Gas phase O_2–CO_2 (95:5).

§§ Specific activity expressed as counts/min/mg fatty acid.

§§§ A unit of hexokinase activity is defined as the quantity of enzyme which phosphorylates 1 μmole 2-deoxyglucose in 15 min at 30°. All other enzyme units are defined as the amount of enzyme which reduces or oxidises 1 μmole TPN or TPNH/min at 20°.

glucoses and the incorporation of glucose carbon into fatty acids by mammary gland slices from pregnant rats with those from rats at early and mid-lactation periods. The results summarised in Table II show that insulin causes a small rise in the conversion of carbon-1 of glucose into $^{14}CO_2$ but does not effect either the oxidation of carbon-6 of glucose or the very low rate of incorporation of glucose carbon into fatty acids by slices of mammary glands from pregnant rats. With the onset of lactation, however, the addition of insulin to the incubation medium causes a very marked stimulation in both the oxidation of carbon-1 of glucose and the incorporation of carbon-6 of glucose into fatty acids. There is a concomitant fall in the oxidation of carbon-6 of glucose. These changes are clearly seen in the C-1/C-6 quotient which is about 4 times greater in the presence of insulin. These effects are apparent both at early (4th day) and at mid-lactation (13th day).

TABLE II

EFFECT OF INSULIN ON THE CONVERSION OF SPECIFICALLY LABELLED GLUCOSES TO $^{14}CO_2$ AND FATTY ACIDS BY MAMMARY GLAND SLICES FROM PREGNANT AND LACTATING RATS

Functional stage	*No. of animals*	*Insulin content of medium*	*% conversion of labelled glucose to $^{14}CO_2$**		*$\frac{^{14}CO_2 \text{ from } G\text{-}1\text{-}^{14}C}{^{14}CO_2 \text{ from } G\text{-}6\text{-}^{14}C}$*	*Specific activity** of fatty acids derived from*	
			[1-^{14}C]glucose	*[6-^{14}C]glucose*		*G-1-^{14}C*	*G-6-^{14}C*
Pregnant	10	0	1.66 ± 0.19	0.98 ± 0.17	1.69	Too low to be determined	
(15th–20th day)		1 i.u	2.82 ± 0.31	1.19 ± 0.17	2.36		
			$p = 0.005$	$p = 0.392$			
Lactating	5	0	7.53 ± 1.05	2.00 ± 0.23	3.77	20 ± 6	79 ± 10
(4th day)		1 i.u.	18.38 ± 1.35	1.19 ± 0.17	15.44	50 ± 9	147 ± 19
			$p < 0.001$	$p = 0.031$		$p = 0.027$	$p = 0.017$
Lactating	6	0	18.17 ± 1.53	2.34 ± 0.44	7.76	120 ± 22	297 ± 46
(13th day)		1 i.u.	26.55 ± 1.86	0.97 ± 0.14	27.37	216 ± 33	535 ± 78
			$p = 0.007$	$p = 0.020$		$p = 0.047$	$p = 0.048$

* 500 mg mammary gland slices incubated for 1.5 h at 37° in 4.5 ml Krebs-Ringer bicarbonate solution with 20 mg glucose containing 0.2 μC ^{14}C in the form of [1-^{14}C]glucose or [6-^{14}C]glucose. Gas phase O_2–CO_2 (95 : 5). Results are expressed as mean ± S.E.N.

** Specific activity expressed as counts/min/mg fatty acid.

The present results confirm and extend the previous observations of BALMAIN *et al.*[23] and ABRAHAM *et al.*[3] on the effect of insulin on fully lactating rat mammary gland slices and, further, they are in keeping with the work of BALMAIN AND FOLLEY[24] who have evidence from studies of the respiratory quotients that insulin does not stimulate the synthesis of fatty acids from glucose and acetate by mammary gland slices from pregnant rats.

Effect of artificial electron acceptors on the oxidation of specifically labelled glucoses

The possibility that the electron transporting system was a rate limiting step regulating the activity of the pentose phosphate pathway in the mammary gland was examined by use of the artificial electron acceptors phenazine methosulphate, pyocyanine and methylene blue (see Table III). While all three of these compounds caused a preferential stimulation of the oxidation of carbon-1 of glucose by slices of lactating mammary gland, phenazine methosulphate was the most active. When

TABLE III

EFFECT OF ARTIFICIAL ELECTRON ACCEPTORS ON THE CONVERSION OF SPECIFICALLY LABELLED GLUCOSES TO $^{14}CO_2$ AND FATTY ACIDS BY MAMMARY GLAND SLICES FROM PREGNANT AND LACTATING RATS

Functional stage	*Compound added (final M concn.)*	*% conversion of labelled glucose to $^{14}CO_2$**		*$^{14}CO_2$ from G-1-^{14}C / $^{14}CO_2$ from G-6-^{14}C*	*Specific activity** of fatty acids derived from*	
		[1-^{14}C]glucose	*[6-^{14}C]glucose*		*G-1-^{14}C*	*G-6-^{14}C*
Pregnant 20 days	None	1.11	0.97	1.14	Specific activity too low to be determined	
	Phenazine $1 \cdot 10^{-4}$	11.97	2.55	4.68		
	Pyocyanin $1 \cdot 10^{-4}$	2.24	1.21	1.85		
	Methylene blue $1 \cdot 10^{-4}$	4.12	1.64	2.52		
Lactating 10 days	None	11.22	2.71	4.14	48	160
	Phenazine $1 \cdot 10^{-5}$	18.20	1.59	11.41	28	120
	Phenazine $1 \cdot 10^{-4}$	33.80	1.32	25.65	51	207
	Phenazine $1 \cdot 10^{-3}$	16.68	6.67	2.50	14	35
	None	12.48	3.15	3.97	20	148
	Pyocyanin $1 \cdot 10^{-4}$	16.92	3.26	5.19	44	84
	Pyocyanin $1 \cdot 10^{-3}$	17.24	2.56	6.74	—	—
	None	12.18	2.61	4.66	22	178
	Methylene blue $1 \cdot 10^{-5}$	14.10	3.20	4.40	32	176
	Methylene blue $1 \cdot 10^{-4}$	16.25	3.26	4.97	57	98
	Methylene blue $1 \cdot 10^{-3}$	14.60	2.14	6.83	15	30

* 500 mg mammary gland sliees incubated for 1.5 h at 37° in 4.5 ml Krebs-Ringer bicarbonate solution with 20 mg glucose containing 0.2 μC ^{14}C in the form of [1-^{14}C]glucose or [6-^{14}C]glucose. Gas phase O_2–CO_2 (95:5).

** Specific activity expressed as counts/min/mg fatty acid.

TABLE IV

EFFECT OF PHENAZINE METHOSULPHATE ON THE OXIDATION OF SPECIFICALLY LABELLED GLUCOSES BY MAMMARY GLAND SLICES FROM PREGNANT AND LACTATING RATS AND BY ADIPOSE TISSUE

Results are expressed as mean ± S.E.M.

Tissue	*Functional stage*	*No. of animals*	*Phenazine methosulphate (1×10^{-4} M final concn.)*	*% conversion of labelled glucose to $^{14}CO_2$*		*$^{14}CO_2$ from G-1-^{14}C / $^{14}CO_2$ from G-6-^{14}C*
				[1-^{14}C]glucose	*[6-^{14}C]glucose*	
Mammary gland*	Pregnant 20 days	9	—	1.56 ± 0.22	0.84 ± 0.09	1.86
			+	8.13 ± 0.63	1.60 ± 0.25	5.08
				$p < 0.001$	$p = 0.019$	
	Lactating 11 days	6	—	11.92 ± 1.68	1.89 ± 0.29	6.31
			+	27.54 ± 2.50	1.13 ± 0.18	24.37
				$p < 0.001$	$p = 0.052$	
Adipose**		7	—	0.58 ± 0.03	0.25 ± 0.04	2.32
			+	2.60 ± 0.26	0.64 ± 0.09	4.06
				$p < 0.001$	$p = 0.002$	

* 500 mg mammary gland slices; ** 250 mg portions of adipose tissue; incubated for 1.5 h at 37° in 4.5 ml Krebs-Ringer bicarbonate medium with 20 mg glucose containing 0.2 μC ^{14}C in the form of [1-^{14}C]glucose or [6-^{14}C]glucose. Gas phase O_2–CO_2 (95:5).

these compounds were present in high concentration in the medium ($1 \cdot 10^{-3}$ *M*) they produced a marked inhibition of fatty acid synthesis.

It is of interest that $1 \cdot 10^{-4}$ *M* phenazine methosulphate greatly stimulates the formation of $^{14}CO_2$ from carbon-1 glucose by mammary gland slices from pregnant rats (Tables III and IV), increases of up to tenfold have been observed and the rate of oxidation of carbon-1 of glucose was often raised to that found in slices of mammary tissue from fully lactating animals. This is in marked contrast to the effect of insulin which produced only a small rise (Table II and Fig. 2). This dye also causes a significant increase in the rate of oxidation of carbon-6 of glucose by tissue slices from pregnant animals suggesting that the electron transport system may be limiting the rate of oxidation via the tricarboxylic acid cycle.

The action of phenazine methosulphate on the oxidation of specifically labelled glucoses by adipose tissue is also shown in Table IV. Adipose tissue resembles mammary gland in that insulin causes an increased $^{14}CO_2$ production from [1-^{14}C]glucose[25]. Phenazine methosulphate produced a four fold and pyocyanin a two fold increase in the oxidation of carbon-1 of glucose. Both these artificial electron acceptors stimulated to a lesser extent the oxidation of carbon-6 of glucose.

Effect of artificial electron acceptors on phosphoglucose isomerase activity

Although the stimulation of carbon-1 oxidation by dyes suggests that one of the rate limiting steps may be the re-oxidation of TPNH, the relative effectiveness of these dyes does not appear to be related entirely to their oxidation-reduction potentials (see DICKENS AND MCILWAIN[26]). There is some evidence based on the rate of oxidation of G-6-P and F-6-P by a yeast preparation, that phosphoglucose isomerase might be inhibited by the use of phenazine methosulphate[26]. However, using the soluble fraction of a mammary gland homogenate, no difference was observed between the rates of oxygen uptake with G-6-P or F-6-P when this carrier was used.

The phosphoglucose isomerase activity of mammary gland homogenates was determined in the presence and absence of $1 \cdot 10^{-3}$ *M* phenazine methosulphate using the colorimetric method described previously[13]. No significant changes were found, the average units of enzyme activity/g tissue from three experiments were 246 in the absence and 222 in the presence of the dye. The differences between the present results and those of DICKENS AND MCILWAIN[26] may perhaps be accounted for in terms of the different enzyme preparations used.

Effect of uncoupling agents on the oxidation of labelled glucoses

To determine whether the stimulatory effects of these dyes could be attributed to an uncoupling of phosphorylation rather than their serving as electron acceptors, lactating mammary gland slices were incubated with varying concentrations of DNP or dicoumarol in the presence of glucose labelled in positions 1 or 6. The results of these experiments are shown in Table V. No preferential stimulation of carbon-1 oxidation was observed and, in fact, with high concentrations of DNP and dicoumarol, which completely uncouple oxidative phosphorylation, a marked inhibition occurred. A two fold increase was observed in the oxidation of carbon-6 of glucose in the presence of $5 \cdot 10^{-6}$ *M* DNP and a four fold stimulation with a similar concentration of dicoumarol. This concentration of DNP was used in the majority of these experiments since it is known to cause approximately 50 % uncoupling of oxidative phos-

TABLE V

EFFECT OF UNCOUPLING AGENTS ON THE CONVERSION OF [1-^{14}C]GLUCOSE AND [6-^{14}C]GLUCOSE TO $^{14}CO_2$ BY LACTATING RAT MAMMARY GLAND SLICES

Results are expressed as mean ± S.E.M. Fisher's P values are given for the comparison of the groups treated with $5 \cdot 10^{-4}$ *M* 2,4-dinitrophenol and $5 \cdot 10^{-4}$ *M* dicoumarol with their corresponding control groups.

Uncoupling agent (final M concn.)		*No. of animals*	*% conversion of labelled glucose to $^{14}CO_2$**		$\frac{^{14}CO_2 \text{ from G-1-}^{14}C}{^{14}CO_2 \text{ from G-6-}^{14}C}$
			[1-^{14}C]glucose	*[6-^{14}C]glucose*	
None		5	15.70 ± 1.57	1.91 ± 0.37	8.22
2,4-dinitrophenol	$2 \cdot 10^{-5}$	2	16.20	2.90	5.59
	$5 \cdot 10^{-5}$	4	15.84 ± 0.35	3.74 ± 0.58	4.32
			$p = 0.635$	$p = 0.033$	
	$1 \cdot 10^{-4}$	2	5.78	1.09	5.31
None		4	19.19 ± 0.35	2.23 ± 0.14	8.61
dicoumarol	$2 \cdot 10^{-5}$	2	14.30	5.88	2.43
	$5 \cdot 10^{-5}$	4	12.72 ± 1.55	8.07 ± 0.38	1.57
			$p = 0.010$	$p < 0.001$	
	$1 \cdot 10^{-4}$	2	4.77	2.54	1.87
	$2 \cdot 10^{-4}$	2	1.83	1.82	1.00

* 500 mg mammary gland slices (11th day of lactation) incubated for 1.5 h at 37° in 4.5 ml Krebs-Ringer bicarbonate solution with 20 mg glucose containing 0.2 μC ^{14}C in the form of [1-^{14}C]glucose or [6-^{14}C]glucose. Gas phase O_2: CO_2(95 : 5).

phorylation[27]. The resultant effect of these changes is a lowering of the C-1/C-6 quotient. Thus it would appear that the stimulatory effects observed with the artificial electron acceptors on the oxidation of carbon-1 of glucose could not be attributed to an increased availability of phosphate or phosphate acceptors by uncoupling oxidative phosphorylation.

These findings are in keeping with those of RACKER[28] and CAHILL *et al.*[6] who studied the effect of DNP on the oxidation of specifically labelled glucoses by ascites tumour cells and liver slices respectively. However, WENNER *et al.*[8], studying Ehrlich ascites tumour cells, found that the oxidation of both carbon-1 and carbon-6 was stimulated equally by the addition of DNP.

No very marked effect on the incorporation of labelled carbon from [1-^{14}C]-glucose and [6-^{14}C]glucose into fatty acids was observed until the concentration of DNP reached $1 \cdot 10^{-4}$ *M* when the synthesis was inhibited by approximately 50%; dicoumarol ($2 \cdot 10^{-4}$ *M* final concentration) also caused a very marked inhibition. These results are in accord with those of TERNER[29] who has shown an inhibitory effect of *p*-nitrophenol on the synthesis of fatty acids in homogenates of mammary gland.

Effect of glucose concentration

The regulatory function exerted by the glucose concentration of the medium on the operation of alternative pathways of glucose oxidation in the lactating rat mammary gland has also been investigated using specifically labelled glucoses; the results of these experiments are shown in Table VI and Fig. 1. As the glucose concentration of the medium was increased from 2 μmoles/ml the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose rose in direct proportion until the concentration reached about 20 μmoles/ml. The oxidation of carbon-6 of glucose remained constant and appeared to be relatively independent of the glucose concentration. The effect of glucose concentration on the two alternative pathways of oxidation is clearly seen in the C-1/C-6 quotient which increases from an average value of about 1.6 to 13.6 when the glucose content of the medium is increased from 2 to 20 μmoles/ml.

The incorporation of glucose carbon into fatty acids is also shown in Table VI. The specific activity of the mixed fatty acids increases as the glucose concentration of the medium is raised. Both the oxidation of carbon-1 of glucose and the incorpora-

TABLE VI

EFFECT OF GLUCOSE CONCENTRATION IN THE MEDIUM ON THE FORMATION OF $^{14}CO_2$ AND FATTY ACIDS FROM [1-^{14}C]GLUCOSE AND [6-^{14}C]GLUCOSE BY LACTATING RAT MAMMARY GLAND

*Initial glucose concentration** *(μmoles/ml medium)*	*% conversion of labelled glucose to $^{14}CO_2$*		*$\frac{^{14}CO_2 \text{ from G-1-}^{14}C}{^{14}CO_2 \text{ from G-6-}^{14}C}$*	*Specific activity** of fatty acids from*	
	[1-^{14}C]glucose	*[6-^{14}C]glucose*		*G-1-^{14}C*	*G-6-^{14}C*
2	28.3	21.9	1.29	11	47
5	27.4	11.7	2.34	20	63
10	24.3	4.97	4.89	46	245
20	34.4	2.48	13.87	100	320
40	16.5	1.08	15.25	85	347
2	27.2	13.4	2.02	40	64
5	23.3	4.8	4.86	—	81
10	21.4	2.23	9.60	64	170
20	16.9	1.28	13.19	74	156
60	4.0	0.55	7.24	70	122

* 500 mg mammary gland slices (15th day of lactation) incubated with 4.5 ml Krebs-Ringer bicarbonate solution for 1.5 h at 37°. The initial glucose concentration of the medium varied from 2 to 60 μmoles/ml. The specific activity of the added glucose was constant at 0.2 μC ^{14}C/20 mg glucose.

** Specific activity expressed as counts/min/mg fatty acid.

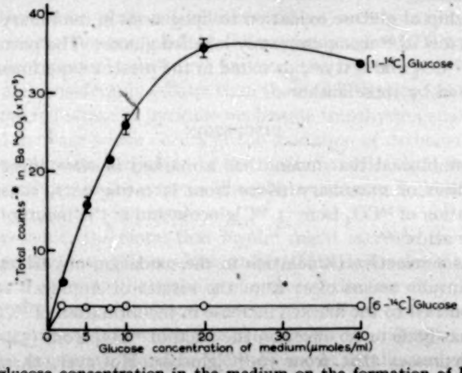

Fig. 1. Effect of glucose concentration in the medium on the formation of $^{14}CO_2$ from [1-^{14}C]-glucose and [6-^{14}C]glucose by lactating mammary gland slices. 500 mg mammary gland slices incubated with 4.5 ml Krebs-Ringer bicarbonate solution for 1.5 h at 37°. The specific activity of the added glucose was constant at 0.2 μC ^{14}C/20 mg glucose.

* A value proportional to the total counts was obtained by multiplying the counts/min of the samples plated at "infinite thickness" by the total weight of $BaCO_3$ expressed in mg. Each point is the mean of five determinations. The vertical lines represent twice the S.E.M.; where no vertical line is given the S.E.M. is too small to show.

tion of labelled glucose into fatty acids appear to reach their maximum values when the glucose concentration is 20 μmoles/ml, the level used in the majority of the experiments reported in this paper.

RACKER[28] has shown that high glucose concentrations inhibited oxidation via the Krebs cycle but increased the oxidation of carbon-1 of glucose by Erhlich ascites tumour cells. The present results on the oxidation of [1-^{14}C]glucose are in agreement with his findings but the operation of the Krebs cycle in the lactating mammary gland appears to be relatively unaffected by the glucose level of the medium. The response of mammary tissue appears to be intermediate between those of Ehrlich ascites tumour cells[28] and liver, since CAHILL *et al.*[6] have shown that the rate of oxidation of both carbon-1 and carbon-6 of glucose by liver is increased as the glucose concentration of the medium is raised. All these experiments were of 60–90 min duration and WENNER *et al.*[8] have reported that the initial rate of $^{14}CO_2$ production from [1-^{14}C]glucose (10 min incubation period) by Ehrlich ascites tumour cells was independent of substrate concentration in the range $2.5 \cdot 10^{-5}$ *M* to $1 \cdot 10^{-2}$ *M*.

The Michaelis constants of the first enzymes of the pentose phosphate pathway and the glycolytic route of the mammary gland are $5.8 \cdot 10^{-5}$ *M* for G-6-P dehydrogenase and $1 \cdot 10^{-3}$ *M* for phosphoglucose isomerase[13] and from this it might be expected that the former pathway would be less dependent upon glucose concentration. The short term experiments of WENNER *et al.*[8] seem to bear this out. Secondary factors arising during the longer periods of incubation may be responsible for the apparent dependence of the pentose phosphate pathway on the glucose concentration in the mammary gland. Since the percentage of the available substrate which is oxidised remains remarkably constant between the concentrations of 2–20 μmoles glucose/ml medium (see Table VI) it would not appear to be simply a case of exhaustion of substrate.

The relationship of glucose oxidation to lipogenesis in mammary tissue has been studied by HIRSCH *et al.*[30] using uniformly labelled glucose. The pattern of change in the formation of $^{14}CO_2$ and fatty acids found in the present experiments are in accord with those reported by these authors.

DISCUSSION

It is now well established that insulin has a marked *in vitro* effect on the glucose metabolism of slices of mammary tissue from lactating rats, causing an increase both in the formation of $^{14}CO_2$ from [1-^{14}C]glucose and in the incorporation of glucose carbon into fatty acids[3, 23].

That there is a selective stimulation in the oxidation of carbon-1 of glucose in the presence of insulin seems clear from the results of ABRAHAM *et al.*[3] who have shown that, in contrast to the marked increase in the formation of $^{14}CO_2$ from [1-^{14}C]-glucose, insulin has little or no effect on the yield of $^{14}CO_2$ from [3,4-^{14}C]glucose and in most cases depresses that from [6-^{14}C]glucose. However, these authors have concluded, on the basis of the incorporation of labelled carbon from [1-^{14}C]glucose and [6-^{14}C]glucose into fatty acids that insulin does not alter the relative proportions of fatty acids derived from the glycolytic and the pentose phosphate pathways, and on these grounds they appear to discount the possibility that insulin preferentially stimulates the pentose phosphate pathway.

In addition to the factors already suggested by ABRAHAM *et al.*[3] and WINEGRAD

AND RENOLD[25] to account for this discrepancy between the data on incorporation of ^{14}C from labelled glucose into CO_2 and fatty acids, there is the possibility that this might be partly explained by the differences in the yields of intermediates for fatty acid synthesis arising from the two pathways of glucose catabolism. The oxidation of three molecules of G-6-P via the pentose phosphate pathway produces only one sixth of the triose phosphate formed by the glycolytic route[31]. It follows that, since a constant balance is maintained in the incorporation of carbon-1 and carbon-6 of specifically labelled glucoses into fatty acids, a considerably larger amount of glucose must be degraded by way of the pentose phosphate pathway. The present work is in part concerned with the selective stimulation of the activity of the pentose phosphate pathway by insulin.

The present experiments show that pretreatment of lactating rats with insulin produced similar effects to those observed *in vitro*. However, no evidence could be found for an increase in the activity of hexokinase, G-6-P dehydrogenase or 6-PG dehydrogenase under these conditions. It can be calculated, from the data in Table I, that the activities of the first two enzymes of the pentose phosphate pathway measured under optimal conditions are much greater, by a factor of about ten, than the actual rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose. This suggests that an increased availability of substrate, either G-6-P or TPN$^+$, is responsible for the stimulation of the activity of the pentose phosphate pathway. It would be interesting to know whether insulin increases the permeability of mammary tissue to sugars as it does in muscle[32], or whether this tissue is freely permeable to glucose as in the case of liver[33].

There is considerable evidence showing that the activity of the pentose phosphate pathway may be partly controlled by the supply of TPN$^+$. The presence of artificial electron acceptors[6] or the supply of substrates which require TPNH for their metabolism[34] can increase the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose by liver slices. Similar effects have been observed in other tissues such as erythrocytes[7], corneal epithelium[5] and ascites tumour cells[8]. In the mammary gland TPN is present almost exclusively in the reduced form[10] and further, the activities of G-6-P and 6-PG dehydrogenases are considerably greater than those of TPNH-cytochrome *c* reductase, TPNH-glutathione reductase or pyridine nucleotide transhydrogenase. This, together with the marked increase which occurs in the oxidation of carbon-1 of glucose when artificial electron acceptors are added to the medium, indicates that in the rat mammary gland the activity of the pentose phosphate pathway is limited by the supply of the oxidised form of the coenzyme.

It seemed possible, therefore, that insulin might increase the supply of TPN$^+$ by stimulating an enzyme system which utilises TPNH. Treatment of lactating rats with insulin has no effect on the activity of either of the two enzymes catalysing the oxidation of TPNH using ferricytochrome *c* or oxidised glutathione as hydrogen acceptors. An enzyme system which has been shown to undergo a marked increase in activity in the mammary gland in the presence of insulin and which is probably TPNH-linked in mammary gland, as it is in liver[35], is that of fatty acid synthesis[4, 36].

The marked difference in the effect of insulin on the oxidation of [1-^{14}C]glucose by tissue slices from pregnant and lactating rats (summarised in Fig. 2) may be related to the different responses of the fatty acid synthesising systems; while insulin greatly increases this process in the lactating mammary gland no such effect has yet been shown on the very low rate of synthesis found in this tissue from pregnant animals. That the activity of the pentose phosphate pathway is limited in mammary gland slices from pregnant rats by the lack of TPN$^+$ is indicated by the six fold increase in the oxidation of carbon-1 of glucose in the presence of phenazine methosulphate which raises the rate up to that found in lactating rats.

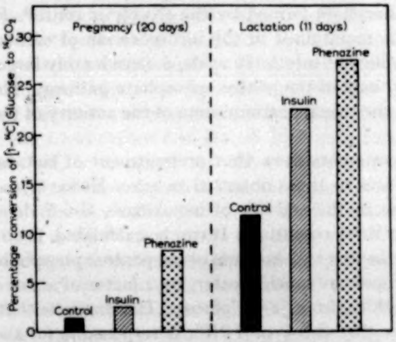

Fig. 2. Relative effects of insulin and phenazine methosulphate on the conversion of [1-^{14}C-] glucose to $^{14}CO_2$ by mammary gland slices from pregnant and lactating rats.

It is suggested, therefore, that the effect of insulin and of phenazine methosulphate in increasing the oxidation of glucose by way of the pentose phosphate pathway is mediated by increasing the reoxidation of TPNH, in the case of phenazine methosulphate by acting as an electron acceptor and of insulin by promoting the utilisation of the hydrogens in the synthesis of long chain fatty acids.

The present experiments show that there is a similarity in the effect of insulin and phenazine methosulphate on the oxidation of carbon-1 of glucose by lactating mammary gland slices and by adipose tissue. Although there is insufficient data to determine whether the increased oxidation of G-6-P is responsible for increased lipogenesis or results from accelerated regeneration of TPN$^+$ when lipogenesis is increased, the effect of artificial electron acceptors lends some support to the latter view. The suggestion of LASFARGUES[37], based on tissue culture studies with mouse mammary gland, that milk fat production is under the specific control of the adipose tissue of the gland is of particular interest in this context. The similar effects of insulin on adipose tissue and mammary gland might perhaps be explained on this basis.

It would appear that the effect of the dyes in promoting the oxidation of glucose by mammary gland slices can be ascribed to their electron acceptor properties rather than to their ability to uncouple oxidative phosphorylation since neither DNP nor dicoumarol caused any perferential stimulation in the oxidation of carbon-1 of glucose, although a marked increase in the oxidation of carbon-6 occurred. Of the dyes tested, phenazine methosulphate proved to be by far the most effective in increasing the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose. The experiments of DICKENS AND McILWAIN[38] are of particular interest in this context. These authors demonstrated that structure was more important than E_h in determining the activity of the carriers for the yeast hexosemonophosphate system, the turnover number of which was highest with flavin enzyme and phenazines. They suggested that the ability to form semiquinones might be an important property common to both these substances. Their system might be taken as a model in attempting to visualise the multienzyme system whereby the hydrogens of the pentose phosphate pathway are utilised for reductive synthetic processes, such as fat synthesis, and their work certainly suggests that a flavin group might be the prosthetic group of the initial acceptor.

ACKNOWLEDGEMENTS

The author is indebted to Professor F. Dickens, F.R.S. for his interest and advice, to Miss P. M. Lovesay for skilled technical assistance and to Dr. K. Hallas-Møller of Novo Terapeutisk Laboratorium, Copenhag[illegible] for a generous supply of crystalline insulin free from glycogenolytic factor. This work has been in part supported by a grant to the Medical School from the British Empire Cancer Campaign.

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THE RELATION OF DIAPHORASE AND CYTOCHROME REDUCTASE

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(Received April 6th, 1959)

SUMMARY

Diaphorase and cytochrome reductase have been extracted differentially from a pig heart-muscle preparation under various conditions. The results indicate that there is no foundation for the suggestion that diaphorase is a degraded form of cytochrome reductase.

INTRODUCTION

In 1938 STRAUB[1] isolated a lemon-yellow fluorescent flavoprotein from a particulate fraction of pig-heart muscle. This flavoprotein was shown[2] to catalyse the oxidation of DPNH with methylene blue as hydrogen acceptor. Later other redox dyes[3] and ferricyanide[4] have been shown to act as acceptors in this reaction, but no physiological acceptors have been described. This flavoprotein was named diaphorase[1] but in the course of time the term diaphorase has come to mean any catalytic oxidation of DPNH or TPNH with artificial acceptors. Considerable confusion has arisen because of this usage; in this communication the term diaphorase is used only to apply to the flavoprotein isolated by STRAUB. Because of the lack of a known physiological acceptor, the role of diaphorase has always remained obscure, but it has been widely assumed that it is the flavoprotein responsible for the physiological oxidation of DPNH, and that some as yet unidentified acceptor links this oxidation to the cytochrome system (see SLATER[5] for a review on this subject). In 1952 MAHLER *et al.*[3] isolated from the same particulate fraction of pig heart another enzyme that catalyses DPNH oxidation. This enzyme, DPNH-cytochrome *c* reductase, can react with the same acceptors as can diaphorase, but unlike the latter, also reacts with cytochrome *c* as electron acceptor. Both enzymes are flavoproteins and both have molecular weights of approximately 80,000[1,3,6]. However, while diaphorase contains no significant quantity of metals, cytochrome reductase contains 4 atoms of non-haem iron per molecule. As the method of STRAUB for the preparation of diaphorase involves several heat treatments, and as it was believed that the iron atoms of cytochrome reductase are essential for catalytic activity[7], it was suggested[8] that diaphorase is probably a degraded form of cytochrome reductase, having lost its iron atoms (and hence its ability to react with cytochrome *c*) during the harsh treatment used in its purification. This interpretation is difficult to accept in view of the known differences in chemical

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The following abbreviations are used; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; cytochrome reductase, DPNH-cytochrome *c* reductase.

and physical properties of the two enzymes (see the review of SLATER[5] for a discussion of these differences); further doubts on the interpretation have arisen from the equivocal nature of the evidence for the role of the metal in cytochrome reductase and other enzymes[9-11]. In view of these uncertainties the possible relation of the two enzymes has been reinvestigated. It has been found possible to extract the two enzymes differentially from pig-heart particles without destruction or apparent interconversion. It is concluded that the two enzymes are distinct entities, not derived one from the other. This conclusion is strengthened by the demonstration that diaphorase is identical with lipoyl dehydrogenase[4]; cytochrome reductase is completely devoid of lipoyl dehydrogenase activity.

MATERIALS AND METHODS

DPNH (92 %) was purchased from the Sigma Chemical Company. Cytochrome *c* was prepared from beef heart by the method of KEILIN AND HARTREE[12] and further purified by calcium phosphate gel column fractionation[13]. Cytochrome reductase activity was measured at 25° by the method of MAHLER *et al.*[3]. Diaphorase activity was measured spectrophotometrically at 420 mμ (1-cm light path) by following the reduction of $K_3Fe(CN)_6$ by enzyme and DPNH. Each spectrophotometer cell contained the following reactants in a total volume of 3.0 ml at 25°; acetate pH 4.8, 500 μmoles; bovine serum albumin 2 mg; $K_3Fe(CN)_6$, 2 μmoles; DPNH, 0.6 μmole. In both assays unit activity is defined as an absorbancy change of 1.0 per min.

Preparation of pig heart particles

This is essentially the same as the method of KEILIN AND HARTREE[14]. Pig hearts were cleaned of fat and connective tissue, minced and the mince washed exhaustively with tap water until the washings were practically colourless. The washed mince, after squeezing through muslin, was suspended in 5 volumes 0.02 *M* Na_2HPO_4 and homogenized for 2 min with a Waring blendor. The homogenate was centrifuged for 15 min at 1400 × *g* and the cloudy supernatant carefully decanted from the rather loose pack of cell debris. The supernatant was adjusted to pH 5.4 by the careful addition of 1 *N* acetic acid and centrifuged at 1400 × *g* for 30 min. The supernatant was discarded, and the precipitate used for the extraction of diaphorase or cytochrome reductase.

Preparation of calcium phosphate gel – cellulose columns

A suspension of 200 ml of Whatman standard grade cellulose powder (10 g/100 ml) was mixed with stirring with 100 ml of a suspension of calcium phosphate gel[15] (30 mg/ml). The mixture was evacuated for 15 min on a water pump to remove dissolved air from solution, and the slurry poured into a column of 2.2 cm diameter. This was allowed to pack by gravity, to give a column about 20 cm in height, with a flow rate of 30–60 ml/h.

RESULTS

Differential extraction of diaphorase and cytochrome reductase with temperature

Heart-muscle preparation was suspended in cold 1 % (w/v) $(NH_4)_2SO_4$ (1 l/heart-muscle preparation from 1 kg wet wt. of washed mince), homogenized for a few seconds to disperse the heart-muscle preparation, and divided into two equal portions. One portion was extracted for 16 h at 4°, the other for 10 min at 38°. This treatment was followed by centrifugation for 1 h at 4100 rev./min in the No. 845 head of a refrigerated International centrifuge. The faintly turbid supernatant was decanted carefully from the residue and the diaphorase and cytochrome reductase activities determined. There is no difference in the amount of diaphorase extracted by these procedures; however, the cytochrome reductase content of the heat extract is some 10 times greater than in the cold extract (*cf.* Table I). This finding is directly opposite to that which would be expected if diaphorase were a degraded form of cytochrome reductase produced by the heat treatment. The two enzymes may be separated quantitatively by fractionation on a calcium phosphate gel column (*cf.* METHODS). After a preliminary fractionation of the cold and hot extracts with $(NH_4)_2SO_4$, the 0.35–0.70-saturation fraction (which contained the bulk of both enzymes) was dialysed overnight against glass distilled H_2O, and then adsorbed on calcium phosphate gel columns. The results are summarised in Fig. 1. Washing with 0.1 *M* phosphate buffer, pH 7.6, resulted in the elution of cytochrome reductase. The apparent diaphorase activity shown by the 0.1 *M* phosphate effluents in the heat-extracted preparation is due to DPNH oxidation by cytochrome reductase (and possibly other flavoproteins) with $K_3Fe(CN)_6$ as acceptor, leaving the diaphorase firmly adsorbed at the top of the column in a lemon-yellow, intensely fluorescent band. After complete elution of the cytochrome reductase the diaphorase was eluted with 0.1 *M* phosphate, pH 7.6, + 5% (w/v) $(NH_4)_2SO_4$. The property of diaphorase of being extremely tightly adsorbed on such columns and only eluted at higher ionic strengths has been used as a method of obtaining the purified enzyme[4,16] without any of the heat treatments previously used in its preparation[1,6]. Diaphorase isolated by this mild procedure has the same properties[16] as that of the enzyme isolated by the method of STRAUB[1] or SAVAGE[6]; hence there is no justification for the suggestion that it is a heat-degradation product of cytochrome reductase.

TABLE I

DIFFERENTIAL EXTRACTION OF DIAPHORASE AND CYTOCHROME REDUCTASE

Enzyme assays were performed as under METHODS. "Diaphorase" activity contains a small contribution from the $K_3Fe(CN)_6$ activity of cytochrome reductase (*cf.* Fig. 1).

Procedure	*Enzyme extracted*	
	"Diaphorase" units/g original mince	*Cytochrome reductase units/g original mince*
Pig heart particles		
Extracted at 4° with 1% $(NH_4)_2SO_4$	3.1	0.13
Extracted at 38° with 1% $(NH_4)_2SO_4$	3.8	1.5
Pig-heart particles, extracted first at 42° with 10% ethanol	0.8, 1.0	4.5, 5.3, 3.2, 5.3, 3.5
Then extracted at 4° with 1% $(NH_4)_2SO_4$	2.4, 3.6	—
Pig-heart particles, extracted first at 4° with 1% $(NH_4)_2SO_4$	8.9, 5.6	—
Then extracted at 42° with 10% ethanol	1.6	13.8, 7.6, 7.5

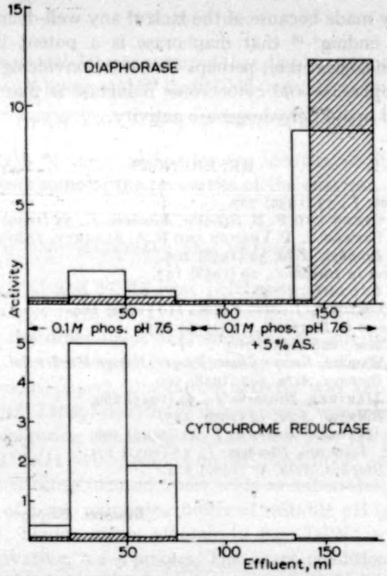

Fig. 1. The differential elution of cytochrome reductase and diaphorase from a calcium phosphate gel–cellulose column. Filled blocks, heart-muscle particles extracted with cold $(NH_4)_2SO_4$; open blocks, extracted with $(NH_4)_2SO_4$ at 38°. The activity is expressed in units/ml. The small "diaphorase" activity eluted from the heat-extracted particles represents the $K_3Fe(CN)_6$ activity of cytochrome reductase.

Sequential extraction of diaphorase and cytochrome reductase

Further evidence that diaphorase is a discrete protein not derived from cytochrome reductase comes from the sequential extraction of the two enzymes from the same sample of the heart-muscle preparation. These results are summarized in Table I. When heart-muscle preparation was treated at 42° with 10 % ethanol to extract cytochrome reductase by the method of MAHLER *et al.*[3], considerable quantities of diaphorase could still be obtained from the residue by extraction with 1 % (w/v) $(NH_4)_2SO_4$ at 4°. In a similar fashion when diaphorase was first extracted from the heart-muscle preparation at 4°, cytochrome reductase could be extracted in excellent yield from the residue by treatment with 10 % ethanol at 42°.

DISCUSSION

The hypothesis that diaphorase was a degraded form of cytochrome reductase appeared unlikely from a comparison of the chemical and physical properties of the two enzymes, as detailed in a review by SLATER[5]. The fact that the two enzymes may be extracted sequentially in normal yields from the same sample of a pig heart-muscle preparation, and the isolation of diaphorase using very mild conditions, provides further evidence against this possibility. The postulation of such a degradation was obviously made because of the lack of any well-defined physiological role of diaphorase. The finding[4,16] that diaphorase is a potent lipoyl dehydrogenase provides such a physiological role; perhaps the most convincing evidence for the unrelated nature of diaphorase and cytochrome reductase is that the latter enzyme is completely devoid of lipoyl dehydrogenase activity.

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THE IDENTITY OF DIAPHORASE AND LIPOYL DEHYDROGENASE*

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(Received April 6th, 1959)

SUMMARY

A method of purification of diaphorase not involving any heat treatments is described. The product is shown to be identical with the enzyme obtained by previous methods of purification. Diaphorase has been found to be a potent lipoyl dehydrogenase; the identity of lipoyl dehydrogenase and diaphorase has been demonstrated by the constant ratio of the two activities during purification, by inhibition studies and by the direct demonstration that reduced diaphorase is rapidly reoxidized by lipoic acid and derivatives. The reaction rates of DPNH oxidation and DPN$^+$ reduction by the enzyme employing a variety of lipoic acid derivatives have been determined. The turnover numbers at 25° vary from 1000 with DL-lipoic acid to about 80,000 with DL-lipoamide. The redox potential of the lipoic acid couple and its variation with pH is also reported.

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* The following abbreviations are used: DPNand DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; lipS$_2$ and lip(SH)$_2$, the oxidized and reduced forms of lipoic acid; PCMS, *p*-chloromercuriphenylsulphonate.

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INTRODUCTION

In previous communications[1, 2] it was reported that diaphorase catalyses the reversible oxdiation of DPNH with lipoic acid or lipoic acid derivatives as hydrogen acceptors.

$$\text{DPNH} + \text{H}^+ + \text{lipS}_2{}^* \rightleftharpoons \text{DPN}^+ + \text{lip(SH)}_2$$

This paper gives details of the evidence for the identity of diaphorase and lipoyl dehydrogenase, and lists some of the properties of the enzyme.

MATERIALS AND METHODS

DPN$^+$ and DPNH (92 %) and PCMS were purchased from the Sigma Chemical Co. DL-Lipoic acid was a gift from Dr. I. C. Gunsalus, DL-lipoamide a gift from Dr. A. F. Wagner, and the other lipoic acid derivatives a gift from Dr. L. J. Reed. Diaphorase activity was measured by following the reduction of $K_3Fe(CN)_6$ as described in the previous paper[5]. Diaphorase activity was also measured at 25° by the method of Savage[3]. Lipoyl dehydrogenase activity was measured spectrophotometrically at 25° by following the change in absorbancy at 340 mμ (1-cm light path) due to oxidation of DPNH (when employing oxidized lipoic acids as substrates) or DPN$^+$ reduction (when using reduced lipoic acids as substrates). The reactions were studied in a volume of 3 ml, containing buffer of suitable pH (generally 150 μmoles of phosphate buffer); bovine serum albumin, 2 mg; DPN$^+$ or DPNH, 0.6 μmole; DL-lipoic acid or derivative, 0.2–5 μmoles. The exact conditions for any particular experiment are given in the text or legends to figures and tables. In all assays unit activity is defined as that amount of enzyme producing an absorbancy change of 1.0 per min.

Preparation of pig heart particles

This was essentially the same as the method of Keilin and Hartree[4]. Details are given in the previous paper[5].

Preparation of calcium phosphate gel-cellulose columns

This was prepared as described in the previous paper[5]. The column was washed with 0.1 *M* phosphate pH 7.6 and allowed to come to temperature equilibrium in a cold room.

Preparation of diaphorase

As shown in the preceding paper[5] diaphorase may be purified (in excellent yield) by a process which avoids the heat treatments previously used in the preparation of this enzyme[3,7]. A pig heart-muscle preparation was suspended in cold 1 % (w/v) $(NH_4)_2SO_4$ (1 l per particle preparation from 1 kg wet wt. of washed mince), homogenized for a few seconds to disperse the particulate preparation and extracted overnight at 4°. The suspension was then centrifuged for 1 h at 4100 rev./min, in the No. 845 head of a refrigerated International Centrifuge or for 20 min at 16,000 rev./min in the No. 21 head of the Spinco Preparative Ultracentrifuge (the latter is the centrifuge of choice as higher yields are obtained).

The extract was then fractionated at pH 7–7.6 (pH obtained by the addition of 1/30 vol. of 0.3 M Na_2HPO_4) with $(NH_4)_2SO_4$, the enzyme being located in the fraction between 0.35 and 0.7 saturation. This fraction was dissolved in cold glass-distilled water and dialysed overnight against 3 l glass-distilled water. The flocculent precipitate formed was centrifuged off and discarded. The orange-coloured supernatant was adjusted to a concentration of 0.1 M phosphate by the addition of 0.3 M phosphate, pH 7.6. This solution was applied to the column and eluted exhaustively with 0.1 M phosphate, pH 7.6. Amber-coloured and colourless impurities are eluted by this buffer, leaving a yellow, fluorescent band at the top of the column. Elution with 0.1 M phosphate was carried out until the E_{280} of the effluent was less than 0.05. (In practice this can be achieved conveniently by washing through about 600–800 ml of buffer overnight from a constant-head device.) The diaphorase was then eluted from the column in a bright-yellow, fluorescent band with 0.1 M phosphate, pH 7.6 containing 4% (w/v) $(NH_4)_2SO_4$. The eluted enzyme was then fractionated with $(NH_4)_2SO_4$, the fraction between 0.55 and 0.7 saturation containing the enzyme in pure or almost pure form. This precipitate was dissolved in 0.03 M phosphate buffer and stored in the frozen state. The enzyme is perfectly stable under these conditions. No loss of activity was observed after storage in the deep freeze for 6 months—in fact appreciable, but varying, activations were observed either on prolonged storage or by incubating at 38°. This phenomenon, which has not yet been extensively investigated, is particularly marked when dichlorophenol indophenol is used as acceptor, and is considered further in Table I.

RESULTS

Identity of the isolated flavoprotein with diaphorase

The identity of the yellow enzyme isolated by the preceding method with that obtained by STRAUB[7] and by SAVAGE[3] is shown in several ways. Firstly, the absorption spectra and described fluorescence characteristics are very similar. Fig. 1 shows the absorption spectrum of the purified enzyme. In the same figure is shown for comparison the spectrum of the enzyme obtained by SAVAGE.

Secondly, the sedimentation constant is the same within experimental error as that obtained by SAVAGE. Fig. 2 shows the Schlieren patterns obtained on examination in the Spinco Model E Ultracentrifuge. Sedimentation coefficients ($S_{20,w}$) determined at concentrations of 7 mg/ml and 4.3 mg/ml were 5.37 and 5.42, respectively, in good agreement with the values given by SAVAGE. The yellow colour is obviously associated with the protein component shown in Fig. 2, as the yellow colour sediments with the only protein component seen.

Thirdly, the activity of the isolated enzyme is in good agreement with that of SAVAGE when tested under her experimental conditions, *i.e.* with dichlorophenol indophenol as acceptor for DPNH oxidation at pH 7.2. Under these conditions the above enzyme had an activity of 4700* units/mg, compared with the value of 5000 units/mg found by SAVAGE.

Fourthly, enzyme prepared by the method of SAVAGE (carried up to Stage VII) exhibited diaphorase activity with $K_3Fe(CN)_6$ as acceptor, with a pH optimum at

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* It should be noted that in distinction to the units of activity in all other assays reported here, the unit of activity reported by SAVAGE represents that amount of enzyme producing an absorbancy change of 0.01/min.

TABLE I

DIAPHORASE AND LIPOYL DEHYDROGENASE ACTIVITIES DURING PURIFICATION

Diaphorase activity using $K_3Fe(CN)_6$ as acceptor was estimated as described under METHODS. Lipoyl dehydrogenase activity with DL-lipoic acid as acceptor was measured as described under METHODS, using a DL-lipoic acid concentration of $6.67 \cdot 10^{-}$ *M*; phosphate, pH 5.9. In each case unit activity is defined as that amount of enzyme producing an absorbancy change of 1.0/min, 1-cm light path.

Procedure	*Protein mg*	*$K_3Fe(CN)_6$ units*	*$K_3Fe(CN)_6$ Specific activity*	*Lipoic acid units*	*$K_3Fe(CN)_6$ activity / lipoic acid activity*
1% $(NH_4)_2SO_4$ extract from 1400 g washed heart muscle	5500	9300	1.7	1220	7.6
Fractionated with $(NH_4)_2SO_4$ As I 0.35–0.75	1660	5190	3.1	650	8.0
Dialysed, fractionated on calcium phosphate — cellulose column, washed with 0.1 *M* phosphate, pH 7.6					
Fractions 1 and 2	1260	350		0	
Eluted with 0.1 *M* phosphate, pH 7.6 + 4% (w/v) $(NH_4)_2SO_4$					
Fractions 3 and 4	43	0		0	
Fraction 5	10	150	15	20	7.5
Fraction 6	94	2200	23.5	264	8.3
Fraction 7	86	2350	27	298	7.9
Fraction 8	30	525	12	66	8.0
Fractions 6 and 7 fractionated with $(NH_4)_2SO_4$					
As II 0–0.55	112	1450	13	185	8.6
As II 0.55–0.70	63	2500	40	305	8.2
Pure enzyme when freshly prepared			40		8.5
after storage for 4 months at —15°			60		8.3
Diaphorase prepared by method of SAVAGE to Stage VII			35		8.9

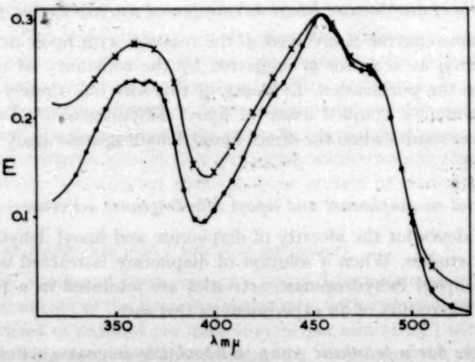

Fig. 1. Comparison of the absorption spectrum of diaphorase (2 mg/ml) prepared by the method of SAVAGE and of this paper. ● present work; × data taken from SAVAGE.

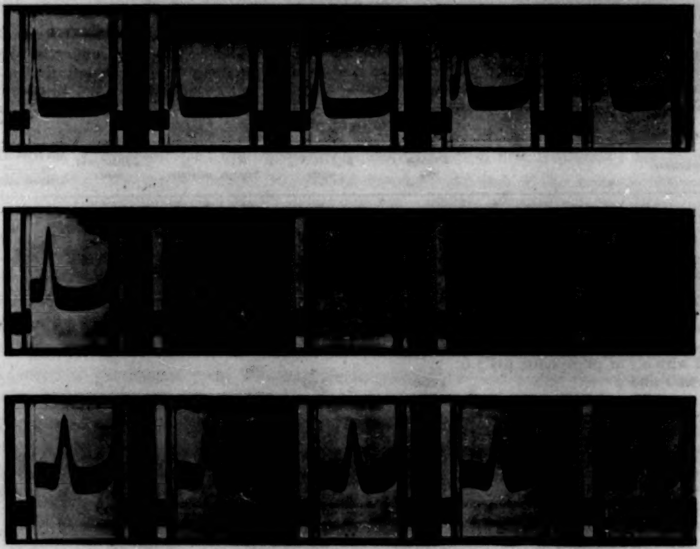

Fig. 2. The sedimentation of diaphorase (10.5 mg/ml) in the Spinco model E ultracentrifuge. The enzyme was in 0.1 *M* phosphate, pH 7.6; average temperature, 17.0°; rotor speed 50,740 rev./min. Photographs were taken at 8-min intervals except for that between the 5th and the 6th photographs, which was an interval of 16 min.

pH 4.8, the same as that found for the enzyme isolated as above. Furthermore, this enzyme exhibited the same ratio of diaphorase to lipoyl dehydrogenase activity as the enzyme described in this paper (Table I).

Constancy of ratio of diaphorase: lipoyl dehydrogenase activity during purification

That the same enzyme is involved in the reaction with lipoic acids as acceptors as with $K_3Fe(CN)_6$ as acceptor is suggested by the constancy of ratio of the two activities during the purification. Evidence of this sort has already been presented using the cytochrome-*c* coupled assay of lipoyl dehydrogenase[1]. Table I shows essentially the same results when the direct lipoyl dehydrogenase assay described under METHODS is used.

Effect of mercurial on diaphorase and lipoyl dehydrogenase activities

Further evidence for the identity of diaphorase and lipoyl dehydrogenase comes from inhibition studies. When a solution of diaphorase is reacted with PCMS both diaphorase and lipoyl dehydrogenase activities are inhibited in a parallel manner. Fig. 3 presents the results of an experiment of this sort.

Involvement of the flavin prosthetic group in lipoyl dehydrogenase activity

When a solution of diaphorase is reduced under anaerobic conditions by $Na_2S_2O_4$

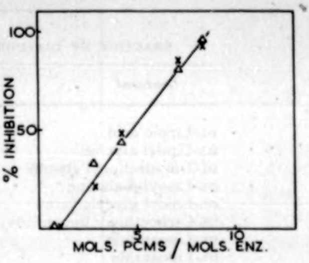

Fig. 3. The effect of PCMS on diaphorase. Enzyme was incubated at 0° in 0.18 M phosphate, pH 5.9, with the amounts of PCMS shown (final concentration of enzyme, $7.1 \cdot 10^{-6}$ M). After 15–20 min, aliquots were removed to determine the rate of DPNH oxidation with either $K_3Fe(CN)_6$ or DL-lipoic acid at 25°. Conditions of assay with both acceptors; phosphate buffer, pH 5.9, 150 μmoles; DPNH, 0.6 μmole; $K_3Fe(CN)_6$, 2 μmoles or DL-lipoic acid, 1 μmole; volume 3 ml. Changes in absorbancy were followed at 420 mμ with $K_3Fe(CN)_6$ and at 340 mμ with DL-lipoic acid (see METHODS). × diaphorase activity; △ lipoyl dehydrogenase activity.

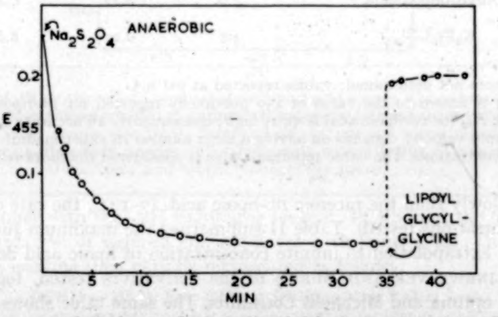

Fig. 4. The involvement of diaphorase flavin in lipoyl dehydrogenase activity. The experiment shown was carried out in an anaerobic spectrophotometer cell (1-cm light path) with two side arms. The main compartment contained 0.06 μmole diaphorase, 150 μmoles phosphate, pH 6.3, in a final volume of 3 ml. Reduction of the enzyme flavin was obtained by tipping 0.4 μmole $Na_2S_2O_4$ from one side arm, and was followed by the decrease in absorbancy at 455 mμ. After 35 min 1.95 μmoles DL-lipoylglycylglycine was added from the other side arm. The immediate return of the yellow colour was also accompanied by return of the fluorescence which had disappeared on the addition of $Na_2S_2O_4$. Temperature, 25°.

there is a complete bleaching of the yellow colour and disappearance of the fluorescence due to this absorption band (Fig. 4). On the anaerobic addition of lipoic acid derivatives (from the side arm of an anaerobic spectrophotometer cell) there is a rapid return of the yellow colour and the fluorescence. This is shown in Fig. 4 for the lipoic acid derivative, lipoyl glycylglycine. Such experiments provide direct evidence for the involvement of the flavin prosthetic group in lipoyl dehydrogenase activity, and coupled with the evidence provided in preceding sections show that the lipoyl dehydrogenase activity is associated with the same protein as that responsible for the diaphorase activity.

Rate of reaction of diaphorase with lipoic acid derivatives

The turnover number of diaphorase as a lipoyl dehydrogenase varies enormously depending on the nature of the lipoic acid substrate, with values of about 1000 (moles of substrate oxidized or reduced per mole enzyme per min at 25°) with DL-lipoic acid to values approaching 100,000 with DL-lipoamide. Samples of the unnatural isomer, (—) lipoic acid, obtained from Dr. A. F. WAGNER and Dr. L. J. REED, reacted very

TABLE II

REACTION OF DIAPHORASE WITH LIPOIC ACID AND DERIVATIVES

Compound	*pH optimum*	*K_m (mM)*	*Maximum turnover number*
DL-Lipoic acid	5.9	2.0	1,000**
DL-Lipoyl glycine	6.3	1.0	1,700
DL-Dihydrolipoyl glycine	7.0	0.7	2,500
DL-Lipoyl β-alanine	*	1.7	25,000
DL-Lipoyl glycylglycine	6.1	1.6	28,000
DL-Carboethoxy lipoanilide	*	about 0.7	about 30,000
DL-Lipoanilide	6.2	0.4	50,000
DL-Lipoamide	6.5	5.0	80,000
DL-Dihydrolipoamide	7.9	0.14	9,400
$K_3Fe(CN)_6$	4.8	0.27	8,000

* pH optimum not determined; values reported at pH 6.3.

** Attention is drawn to the value of 230 previously reported for DL-lipoic acid[1]. As shown in the table the K_m for DL-lipoic acid is quite high; consequently an accurate value for the extrapolated maximum velocity depends on having a large number of experimental values at different lipoic acid concentrations. The value reported above is considered the more reliable.

much more slowly than the racemic DL-lipoic acid. (7–12 % the rate of DL-lipoic acid at the concentrations tested). Table II summarises the maximum turnover numbers (obtained by extrapolation to infinite concentration of lipoic acid derivative by the method of LINEWEAVER AND BURK[8]) of the derivatives tested, together with the observed pH optima and Michaelis Constants. The same table shows for comparison the values obtained for DPNH oxidation with $K_3Fe(CN)_6$ as acceptor (diaphorase activity). It must be emphasised that these values probably do not represent maximal values, as they were all determined at a relatively low concentration of DPNH or DPN+ ($2 \cdot 10^{-4}$ *M*); the variation with DPNH or DPN+ concentration has not yet been determined.

The redox potential of the lipoic acid couple

From variation of the equilibrium constant with pH of the reaction, $DPNH + H^+ + lip\ S_2 \rightleftharpoons DPN^+ + lip(SH)_2$, the variation the redox potentials of the lipoic acid couple with pH have been determined at 25°, making use of the known redox potential[9] of the DPNH–DPN+ couple (—320 mV at pH 7.0, 25°) and its variation with pH of —30 mV per pH unit. The results are shown in Fig. 5, which shows also for comparison the value at pH 7.1 recently reported by SANADI *et al.*[10] and that at pH 6.0 reported by GOLDMAN[11]. As would be expected for a redox couple which involves two hydrogen atoms for reduction but produces no hydrogen ions, the redox potential varies by —60 mV per pH unit (theoretical variation, —59 mV per pH unit at 25°).

DISCUSSION

The constancy of ratio during purification of DPNH dehydrogenase activity using either $K_3Fe(CN)_6$ or lipoic acid as acceptor, leaves little doubt that diaphorase and lipoyl dehydrogenase are identical. This interpretation is strongly substantiated by the finding that both diaphorase and lipoyl dehydrogenase activities are affected in

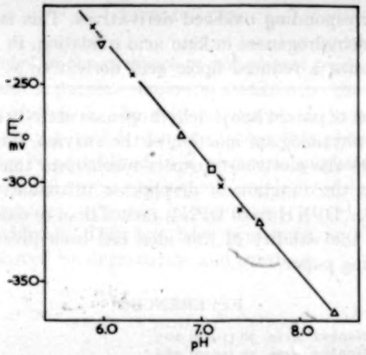

Fig. 5. The effect of pH on the redox potential of the lipoic acid couple. The values were calculated from equilibrium constants determined at various pH values by relating these values to the redox potential of the DPN$^+$ couple (see text). Temperature, 25° except where noted. × DL-lipoylglycine couple; △ DL-lipoamide couple; □ DL-lipoamide couple (value of SANADI *et al.*, 22°); ▽ DL-lipoic acid couple (value of GOLDMAN, 22°).

an identical manner with the –SH combining reagent, PCMS, and the direct demonstration of reoxidation of reduced enzyme-flavin with lipoic acids. The mechanism of action of this enzyme then may be represented as follows:

$$\text{DPNH} + \text{H}^+ + \text{Enz. FAD} \rightleftharpoons \text{DPN}^+ + \text{Enz. FADH}_2$$

$$\text{Enz. FADH}_2 + \text{lip S}_2 \rightleftharpoons \text{Enz. FAD} + \text{lip (SH)}_2$$

The redox potential of the lipoic acid couple reported by SANADI *et al.*[10] and by GOLDMAN[11] and confirmed in this paper is such that its reversible reaction with the FAD couple would be expected.

The rate of reaction of the enzyme with the various lipoic acids tested deserves some comment. The samples of (—) lipoic acid tested contained small amounts of the (+) isomer[12]. It is, therefore, possible that the enzyme may not react at all with the unnatural (—) isomer; the observed rate being due to the presence of some (+) isomer which may equilibrate with the (—) isomer in a non-enzymic reaction

$$(+)\ \text{lip(SH)}_2 + (-)\ \text{lip S}_2 \rightleftharpoons (+)\ \text{lip S}_2 + (-)\ \text{lip(SH)}_2$$

Of much greater interest however is the variation in rate as the nature of the lipoic acid substrate is changed. With the derivatives which still have a charged carboxyl group (lipoic acid, lipoyl glycine, lipoyl β-alanine and lipoyl glycylglycine) the maximum turnover number increases the further the charged carboxyl group is separated from the dithiolane ring. The highest activities are obtained with those structures which are unionized in the oxidized form (lipoanilide, carboethoxylipoanilide, lipoamide). This variation would suggest that the presence of an anionic charge on the substrate hinders approach to the enzyme, and may have some bearing on the naturally occurring coenzyme form of lipoic acid. In this respect it is interesting to note that the detailed work of REED *et al.*[13] indicates that the naturally occurring form of lipoic acid in keto acid oxidation is as a protein-bound form in which the carboxyl group is involved in linkage to the protein. It is also interesting to note that of the reduced lipoic acid derivatives tested, the pH optima are more physiologcal than for the corresponding oxidized derivatives. This is in keeping with the known role of lipoyl dehydrogenase in keto acid oxidation, in which the function of the enzyme is to oxidize a reduced lipoic acid derivative at the expense of DPN reduction[14, 15].

The demonstration of potent lipoyl dehydrogenase activity of diaphorase suggests that this may be the physiological function of the enzyme, rather than the function of a DPNH oxidase in the electron-transport mechanism that has previously been ascribed to it[16]. Thus the function of diaphorase in oxidative metabolism would appear to be to produce DPNH from DPN$^+$, rather than to oxidise DPNH to DPN$^+$. Further evidence for the validity of this idea has been presented[2, 17], and will be detailed in the following paper.

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DIPHENYLENEDIOXIDE-2,3-QUINONE: AN INTERMEDIATE IN THE ENZYMIC OXIDATION OF CATECHOL

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(Received April 7th, 1959)

SUMMARY

A yellow quinone is formed from catechol by oxidation with polyphenol oxidase or iodates. It has been isolated in crystalline form and shown by degradation and synthesis to be diphenylenedioxide-2,3-quinone.

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INTRODUCTION

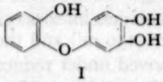
The oxidation of catechol in the presence of polyphenol oxidase has been shown to yield both a yellow and a purple quinone in addition to the three isomeric tetrahydroxydiphenyls (dicatechols)[1]. The yellow pigment (E) produced at a substrate concentration greater than $1 \cdot 10^{-3}\ M$ can be reduced to a phenol (F), $C_{12}H_8O_4$, with ascorbic acid and F can be re-oxidized to E with ceric sulphate or with the enzyme. F contains an *ortho* diphenol group which can be methylated to give the di-O-methyl derivative. It was therefore suggested in a previous paper[1] that F is probably a dihydroxydiphenylenedioxide. This has been confirmed and the structure of the phenol and quinone proved by degradation and synthesis.

RESULTS AND DISCUSSION

The quinone (E) has been isolated from an enzymic oxidation of catechol but better yields are obtained by oxidation with sodium iodate. It is a typical *o*-quinone, easily reduced to an *o*-diphenol (F) and condenses with *o*-phenylenediamine to give a brilliant blue phenazine. The infra-red spectrum of di-O-methyl F shows that only ether-linked oxygen atoms are present[2]. When F is refluxed with a mixture of acetic and hydrobromic acids, catechol can be recovered from the reaction mixture showing that the two aromatic rings are joined by oxygen and not carbon linkages. Cleavage of F with aniline hydrochloride yields a compound not separable on chromatography in three different solvent mixtures from 2,3′,4′-trihydroxy-diphenyl ether (I). 2,3′,4′-Trimethoxy-diphenyl ether was synthesized by condensation of 4-iodoveratrole with potassium guaiacol and demethylated to yield (I) by heating with pyridine hydrochloride.

The quinone (E) is much more labile than F and can be hydrolysed with acids and alkalis and most conveniently with perchloric acid to yield catechol and 2,5-dihydroxy-benzo-1,4-quinone, characterized as 2,5-diethoxy-benzo-1,4-quinone. Methanolysis of E yields catechol and what is probably 4,5-dimethoxy-benzo-1,2-quinone. The isolation of the 1,2,4,5-tetrahydroxy derivatives shows that the oxidative formation of the diphenylenedioxide ring system has occurred through *para* and not *ortho* condensation of the catechol.

Oxidation of 2,3′,4′-trihydroxydiphenyl ether (I) with aqueous silver nitrate yields the diphenylenedioxide-2,3-quinone (E).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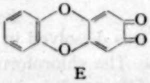
E

This work shows that during the formation of "catechol melanin" by enzymic oxidation of catechol both carbon–oxygen and carbon–carbon linkage occurs.

EXPERIMENTAL

Melting points recorded in this paper represent uncorrected values. *U.v. spectra* were all determined with a Unicam S.P. 500 spectrophotometer.

Preparation of quinone (E)

(1) *By enzymic oxidation.* Catechol (0.025 mole) in 250 ml. McIlvaine's buffer pH 5.1 was stirred with 250 ml of polyphenol oxidase solution, containing 50,000 catecholase units[1] for 10 min at room temperature. The yellow-brown solution was extracted with 2 × 250 ml lots of chloroform. The chloroform layers were combined and washed with 0.25 *M* sodium acetate (100 ml) and dried (Na_2SO_4). The chloroform solution was passed through a column of silica gel (15 × 2.5 cm) which removed phenolic contaminants. The solution could then be shown by paper chromatography to contain only E. The chloroform was removed under reduced pressure and the quinone recrystallized from acetone as orange-red prisms, m.p. 260–261° with decomposition. Yield: 35 mg, 1.3% (found: C, 66.8; H, 2.9; O, 30.1; calc. for $C_{12}H_6O_4$: C, 67.2; H, 2.8; O, 29.9%); ultraviolet max. at 282, 410 and min. at 248, 350 mμ (log ε, 4.13, 2.74, and 3.45, 2.57 respectively).

(2) *By iodate oxidation.* Superior yields of E can be obtained by oxidation of catechol with iodates. Catechol (0.2 mole) was dissolved with stirring in chloroform (2 l) and a solution of sodium iodate (0.2 mole) in water (400 ml) added with continuous stirring. After 10 min "Celite 545" (Johns-Mannville, 50 g) was added to the thick black emulsion and the mixture filtered on a sinter glass funnel. The chloroform layer was removed and the aqueous layer and celite washed with fresh chloroform (150 ml). The combined extracts and washings were shaken with 0.1 *M* sodium thiosulphate (400 ml) to remove iodine, and then with 0.25 *M* sodium acetate (400 ml). The chloroform solution was dried ($CaCl_2$), concentrated under reduced pressure to about 200 ml, and run through a silica gel (B.D.H. "for chromatographic adsorption") column (30 × 3.5 cm). E was eluted with chloroform, concentrated under reduced pressure, precipitated by dilution with light petroleum B.P. 60–80°, and recrystallized from acetone. Yield 1.88 g, 8.7%, m.p. 260–261° unchanged on mixing with the quinone from the enzymic oxidation.

Preparation of phenazine derivative

E (140 mg) in acetic acid (10 ml) was mixed with *o*-phenylenediamine (400 mg) in the same solvent (10 ml). Very dark blue crystalline material was deposited almost immediately and was recrystallized from acetic acid. M.p. > 300°, yield 36 mg. (Found: N, 10.6; calc. for $C_{18}H_{10}N_2O_2$: N, 9.8%. Visible absorption spectrum shows max. at 540, 578, 622 and min. at 550, 596 mμ (log ε, 4.05, 4.92, 5.13 and 4.04, 4.21 respectively).

Reduction of E

(1) E (350 mg) was dissolved in chloroform (150 ml) and the solution saturated with sulphur dioxide. The chloroform was removed under reduced pressure and the residue crystallized from 20% aqueous alcohol containing SO_2. Colourless needles, m.p. 197° decomp. (rapid heating) undepressed by admixture with a sample of compound F[1]. Yield 246 mg 70%.

(2) E (90 mg) was dissolved in 50 ml 90% ethanol containing ascorbic acid (1 g). After decolorisation had occurred, water (4 volumes) was added and the solution extracted with ethyl acetate (2 × 100 ml). The ethyl acetate was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was recrystallized from aqueous ethanol to give F. Yield 67 mg, 74%.

Cleavage of F

Compound F (100 mg) was refluxed with glacial acetic acid (10 ml) and 48% aqueous hydrobromic acid (10 ml) for 10 h. The solution was brought to pH 4 by addition of sodium hydroxide (4.5 g) in water (80 ml) and extracted with ethyl acetate (3 × 25 ml). The combined extracts were taken to small volume and mixed with a small quantity of cellulose powder and allowed to dry. The dry powder was packed on top of a cellulose powder column (2.6 × 2.8 cm) and the column eluted with water. The first 15 ml of eluate was collected and extracted with four successive 0.5 volume of ethyl acetate and the combined extract taken to small volume. Toluene was added and the mixture again concentrated. On standing crystals of catechol formed, m.p. and mixed m.p. 103°, not separable on chromatography from an authentic specimen.

Compound F (10 mg) and aniline hydrochloride (50 mg) were heated in a sealed tube at 210° for 1 h. The black residue was dissolved in water, acidified, and extracted with ethyl acetate. The extract contained only a single phenolic substance which could not be separated from 2,3',4'-trihydroxydiphenyl ether (I) on paper chromatography in water; n-butanol–acetic acid–water (4:1:5); or chloroform–acetic acid (5:3) saturated with water.

Hydrolysis of E

E is hydrolysed by acids and alkalis. E (100 mg) was dissolved in boiling glacial acetic acid (15 ml) and 60% perchloric acid (5 ml) added to the hot solution. The colour immediately changed from a clear orange to a brown yellow. The solution was cooled after 2 min and solid potassium carbonate added to remove the perchloric acid. The solution was diluted with water and extracted 3 times with ethyl acetate (0.5 volume). Paper chromatography indicated the presence of catechol and 2,5-dihydroxy-benzo-1,4-quinone (by co-chromatography with the commercially available compound). A saturated solution of calcium acetate in boiling methanol was added until there was no further precipitation. The purplish precipitate was removed by centrifugation and suspended in water. Concentrated phosphoric acid was added dropwise until the precipitate had dissolved. The solution was extracted 3 times with ethyl acetate (0.5 volume), the extracts dried (Na_2SO_4) and the ethyl acetate removed under reduced pressure. The residue was dissolved in ethanol (10 volumes), saturated with HCl gas, and allowed to stand overnight at 0°. The yellow crystals were filtered off and washed with a few drops of ethanol. Yield 17.5 mg (20%). M.p. 183° undepressed by admixture with an authentic sample of 2,5-diethoxy-benzo-1,4-quinone. (Found: C, 62.3; H, 6.3; calc. for $C_{10}H_{12}O_4$; C, 61.3; H, 6.1%.) Under the same conditions of hydrolysis catechol remains unchanged.

Methanolysis of E

E (200 mg) was dissolved in boiling methanol (100 ml) and HCl gas passed through the solution. The solution was allowed to cool in the refrigerator and the yellow crystals filtered off after 3 days. Yield 108 mg. M.p. 294–297° dec. (Found: C, 56.9; H, 5.0; calc. for $C_8H_8O_4$; C, 57.1; H, 4.7%.)

Synthesis of 2,3',4'-trihydroxydiphenyl ether (I)

Guaiacol (7.5 g) and potassium hydroxide (2.2 g) were mixed and heated *in vacuo* for 2 h at 150° to remove water. Freshly precipitated copper (0.4 g) was successively washed with acetone and ether and heated with guaiacol (2 ml) at 150° for 5 min. The catalyst and 10.6 g of 4-iodoveratrole (prepared by the method of Bruce and Sutcliffe[3]) were added to the dry potassium salt of guaiacol and the mixture stirred and heated under reflux at 180° for 4 h. The reaction mixture was then cooled, broken up and extracted alternately with water and ether (300 ml each total). The extracts were combined, shaken, and the ether layer removed. The aqueous layer was re-extracted with ether and the combined ether extracts filtered to remove the catalyst and extracted with dilute sodium hydroxide solution to remove guaiacol. The ether was then dried (Na_2SO_4) and removed *in vacuo*. The residue, a brown gum, was steam-distilled to recover unchanged 4-iodoveratrole (3 g). The semi-solid residue was crystallized from 50% aqueous alcohol and after repeated crystallization gave 2.1 g (28% yield) of 2,3',4'-trimethoxydiphenyl ether, m.p. 72–73°. (Found: C, 68.5; H, 6.1; OCH_3, 35.5; calc. for $C_{12}H_7O(OCH_3)_3$: C, 69.3; H, 6.1; OCH_3, 35.7%.) The trimethoxy diphenyl ether (1.5 g) was mixed with freshly prepared pyridine hydrochloride (7 g) and heated at 210° for 2 h. The reaction mixture was cooled, dissolved in water, and extracted with 6 × 50 ml portions of ether. The combined ether extract was dried (Na_2SO_4) and taken to dryness *in vacuo*. The residual gum crystallized on rubbing with a glass rod and was re-crystallized from toluene giving clusters of needles of the trihydroxydiphenyl ether (930 mg 74% yield). M.p. 138°. (Found: C, 65.8; H, 4.7; calc. for $C_{12}H_{10}O_4$: C, 66.0; H, 4.6.)

2,3-Dihydroxydiphenylenedioxide (*F*)

Trihydroxydiphenyl ether (400 mg) was dissolved in water (75 ml) containing potassium acetate (1 g) and mixed with a solution of silver nitrate (1.24 g: 4 mol. prop.) in water (15 ml). After stirring for 1 h at 25° the black precipitate was filtered off. The filtrate was extracted with ether and the precipitate washed with ethanol until all the yellow colour was extracted. The ether and alcohol extracts, which were shown by paper chromatography to contain E and F, were combined, saturated with sulphur dioxide, and concentrated to small volume. Addition of water and cooling caused the separation of crystals of 2,3-dihydroxydiphenylenedioxide (270 mg, 68%). Recrystallization from 20% aqueous alcohol containing SO_2 yielded colourless felted needles, m.p. 197° decomp. (rapid heating) undepressed by admixture with a sample of compound F. The dihydroxydiphenylenedioxide was methylated with diazomethane giving a quantitative yield of the dimethyl ether m.p. 110°. The m.p. is undepressed by admixture with a sample of the dimethyl ether of compound F.

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SOME ACTIONS OF THYROXINE ON OXIDATIVE PHOSPHORYLATION

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(Received April 2nd, 1959)

SUMMARY

The effects of thyroxine and three of its analogues on oxidative phosphorylation have been tested using intact mitochondria and submitochondrial particles.

When intact mitochondria were maintained in isotonic sucrose, thyroxine was virtually without effect unless the mitochondria were preincubated, for a minute or more, with the reaction medium in the absence of substrate. Under such conditions phosphorylation was uncoupled from the oxidation of succinate.

Intact mitochondria washed with hypotonic phosphate buffer lost oxidative activity and exhibited poorer phosphorus to oxygen ratios than aliquots of the mitochondrial suspension maintained in isotonic sucrose. Both the oxidation rate and the phosphorus to oxygen ratio of the phosphate-washed mitochondria were stimulated by thyroxine.

Submitochondrial particles prepared from sonic extracts of mitochondria also showed an improvement in the rate of oxidation and in the phosphorus to oxygen ratios in the presence of thyroxine or triiodothyronine. This was true when either succinate or reduced diphosphopyridine nucleotide were used as substrates.

Thyroxine concentrations from 2 to 10 times those necessary to produce increases in rate of oxidation or phosphorus to oxygen ratios uncoupled phosphorylation from oxidation in submitochondrial particles preincubated with the reaction medium for 2 min prior to the addition of thyroxine and substrate.

Thyroxine appeared to have little effect on the adenosinetriphosphatase activity or on the rate of exchange of inorganic phosphate with the terminal phosphate of adenosinetriphosphate in submitochondrial particles.

INTRODUCTION

A number of investigations[1-3] have shown that under certain circumstances thyroxine will uncouple phosphorylation from oxidation in whole mitochondria prepared from rat liver. It was felt that such an action of thyroxine might account for the increase in oxidative rate caused by the thyroid hormones *in vivo*. TAPLEY, COOPER AND LEHNINGER[4] have reported that thyroxine had no effect on oxidative phosphorylation in the digitonin–enzyme complex derived from mitochondria. Some pre-treatment of

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the mitochondria has usually been required in order to demonstrate uncoupling, and no instances of direct stimulation of oxidation in the absence of uncoupling were reported. The previous studies have all been carried out using the Warburg apparatus to measure oxygen uptake. The present study was, therefore, undertaken to re-examine the effects of thyroxine on mitochondria and submitochondrial particles using the Clarke oxygen electrode to measure oxygen uptake. The use of this technique made it possible to measure the initial rates of oxygen uptake accurately and to use incubation times of the order of 1 or 2 min. As reported in a preliminary communication[5] thyroxine and triiodothyronine were found to increase the rate of oxidation of succinate and DPNH at the submitochondrial level. The rise in oxidation rate was accompanied by an increase in the efficiency of phosphorylation. Similar results were obtained with whole mitochondria washed in hypotonic phosphate buffer. After suitable pretreatment of either intact mitochondria or submitochondrial particles thyroxine uncoupled oxidative phosphorylation, but this did not result in a rise in the oxidative rate. Higher concentrations of thyroxine were necessary for uncoupling than for the stimulation of oxidation and phosphorylation. The purpose of this paper is to describe these observations in detail and to discuss the possible relationship between them and the actions of the thyroid hormones in intact animals.

MATERIALS AND METHODS

Mitochondria were prepared from rat liver by the method of KIELLEY AND KIELLEY[6]. The methods used for preparing submitochondrial particles and for measuring oxygen uptake with the Clarke oxygen electrode have been described earlier[7]. Phosphorylation and [^{32}P]ATP exchange were measured using the ^{32}P incorporation method of NIELSEN AND LEHNINGER[8]. ATPase activity was determined by the method of KIELLEY AND KIELLEY[6].

Thyroxine was used as the sodium salt. Triiodothyronine, diiodothyronine, and tetrachlorothyronine were generously supplied by Dr. J. WOLFF. All biochemicals were obtained commercially, ADP as the sodium salt and DPNH as the enzymically prepared material.

RESULTS

Influence of thyroxine on submitochondrial particles

Table I shows representative data on the effects of thyroxine on oxidation and phosphorylation with submitochondrial particles. For each substrate tested thyroxine increased the rate of oxygen uptake and the P/O ratio, unless the particles were pre-incubated with the reaction medium prior to the addition of thyroxine and substrate. Preincubation of the particles abolished the stimulation of oxygen uptake and changed the effect of thyroxine on the P/O ratio from a stimulatory to an inhibitory one. In all of the incubations described in this paper the submitochondrial particles were equilibrated with thyroxine in the reaction medium for 30 sec prior to the addition of substrate.

The following abbreviations are used: ATP for adenosine triphosphate; ADP for adenosine diphosphate; AMP for adenosine-5′-phosphate; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; DNP for 2,4-dinitrophenol; and P for inorganic orthophosphate.

TABLE I

THE INFLUENCE OF THYROXINE ON SUBMITOCHONDRIAL PARTICLES

Conditions: 10 µmoles ADP, 10 µmoles AMP, 10 µmoles $MgCl_2$, 20 µmoles PO_4, pH 7.0, $1 \cdot 10^6$ counts/min ^{32}P, where used 5 µmoles succinate, 2.5 µmoles DPNH, or 20 µmoles β-hydroxybutyrate and 1 µmole DPN^+; Preparation No. 1: 0.09 mg protein nitrogen, Preparation No. 2: 0.16 mg protein nitrogen; 1.9 ml total volume; 28°.

Preparation	*Substrate*	*Thyroxine present (final concn.)*	*Preincubation min*	*Oxygen uptake* (µatoms O/min)	*Oxygen uptake* % control	*P/O ratio*	*P/O ratio* % control
No. 1	Succinate	None	0	0.098	—	0.59	—
	Succinate	$2.5 \cdot 10^{-5}$ *M*	0	0.137	140	0.70	119
	Succinate	None	2	0.062	—	0.55	—
	Succinate	$2.5 \cdot 10^{-5}$ *M*	2	0.064	103	0.47	81
No. 2	Succinate	None	0	0.184	—	0.59	—
	Succinate	$2.5 \cdot 10^{-5}$ *M*	0	0.235	128	0.77	131
	DPNH	None	0	0.376	—	0.65	—
	DPNH	$2.5 \cdot 10^{-5}$ *M*	0	0.470	125	0.77	118
	β-hydroxybutyrate + DPN^+	None	0	0.205	—	0.97	—
	β-hydroxybutyrate + DPN^+	$2.5 \cdot 10^{-5}$ *M*	0	0.252	123	1.08	111

Influence of thyroxine on whole mitochondria

The influence of thyroxine on the initial rates of oxidation and phosphorylation was also studied with intact mitochondria. Table II shows some typical results. When the effects of thyroxine were studied using freshly prepared mitochondria suspended in isotonic sucrose, a small increase in P/O ratio was noted, but this was accompanied by little change in the rate of oxygen uptake. Preliminary results[5] had indicated that the increase in P/O ratio in the presence of thyroxine was accompanied by a drop in the rate of oxygen uptake but more extensive experiments have shown that this is not generally the case. Table II also indicates that if the mitochondria were washed and resuspended in hypotonic phosphate buffer (0.03 *M*, pH 7.0), thyroxine markedly increased the rate of oxidation and the P/O ratio in a manner comparable

TABLE II

THE INFLUENCE OF THYROXINE ON WHOLE MITOCHONDRIA

Conditions: 2 µmoles ADP, 15 µmoles AMP, 20 µmoles PO_4, pH 7.0, $1 \cdot 10^6$ counts/min ^{32}P, 5 µmoles succinate, and 200 µmoles sucrose in incubations of mitochondria suspended in isotonic sucrose; 0.63 mg mitochondrial nitrogen; 1.9 ml total volume; 28°.

Mitochondrial suspension medium	*Thyroxine present (final concn.)*	*Preincubation min*	*Oxygen uptake* (µatoms O/min)	*Oxygen uptake* % control	*P/O ratio*	*P/O ratio* % control
Isotonic sucrose	0	0	0.385	—	1.37	—
Isotonic sucrose	$5 \cdot 10^{-6}$ *M*	0	0.389	101	1.53	112
Isotonic sucrose	$2.5 \cdot 10^{-5}$ *M*	0	0.393	102	1.49	109
Isotonic sucrose	0	1	0.333	—	1.35	—
Isotonic sucrose	$2.5 \cdot 10^{-5}$ *M*	1	0.257	78	0.57	42
0.03 *M* phosphate	0	0	0.236	—	0.72	—
0.03 *M* phosphate	$5 \cdot 10^{-5}$ *M*	0	0.371	157	0.85	118
0.03 *M* phosphate	0	1	0.208	—	0.64	—
0.03 *M* phosphate	$5 \cdot 10^{-5}$ *M*	1	0.213	102	0.24	44

with that observed using submitochondrial particles. Since the same amount of mitochondrial nitrogen was present in all of the incubations in Table II it is apparent that washing the mitochondria with hypotonic phosphate buffer caused a decrease in the rate of oxidation and the P/O ratios of the controls. Addition of thyroxine restored the rate of oxidation to the level found before washing with phosphate. The P/O ratio of the phosphate washed mitochondria was increased substantially by thyroxine but not to the control value obtained with the mitochondria suspended in isotonic sucrose.

Preincubation of either suspension of mitochondria with the reaction medium for a period of time sufficient to exhaust most of the endogenous substrate (1 min in these experiments) resulted in greatly reduced P/O ratios when thyroxine and substrate were added. The controls in which substrate alone was added after the preincubation period were unaffected. The lower P/O ratios obtained with preincubated mitochondria in the presence of thyroxine were often accompanied by lower rates of oxidation. In no case was the rate of oxidation increased when such uncoupling occurred. It was difficult to obtain reproducible P/O ratios with intact mitochondria suspended in hypotonic phosphate buffer since even at 0° a significant drop in P/O ratio could be observed in the hour or less between the first incubation and the final one. The control values shown in Table II were always measured before those in which thyroxine was present. Therefore, the P/O ratios that are given for the controls may be slightly higher than they would have been if the controls had been run at precisely the same time as the incubations in which thyroxine was present. No such difficulty was encountered with the suspensions of submitochondrial particles which were stable with respect to the rate of oxidation and P/O ratio for at least 2 to 3 h after preparation.

Influence of thyroxine analogues on submitochondrial particles

Table III gives a comparison of the influence of thyroxine and of three thyroxine analogues on the rate of oxidation and P/O ratio using submitochondrial particles. Only triiodothyronine approached the activity of thyroxine, and it was not as effective.

TABLE III

THE INFLUENCE OF THYROXINE ANALOGUES ON SUBMITOCHONDRIAL PARTICLES

Conditions: 5 μmoles succinate as substrate, 0.18 mg protein nitrogen; other conditions as in Table I.

Additions	*Oxygen uptake*		*P/O ratio*	
	(μatoms O/min)	*% control*		*% control*
None	0.124	—	0.35	—
$5 \cdot 10^{-5} M$ thyroxine	0.209	169	0.53	151
$5 \cdot 10^{-5} M$ triiodothyronine	0.197	159	0.47	134
$5 \cdot 10^{-5} M$ diiodothyronine	0.154	124	0.40	114
$5 \cdot 10^{-5} M$ tetrachlorothyronine	0.154	124	0.35	100

Time course of oxygen uptake

Fig. 1 shows the time course of oxygen uptake by submitochondrial particles oxidizing succinate. The curves were drawn directly from oxygen electrode traces made with successive incubations of aliquots of the same submitochondrial particle preparation. The broken line is from the control trace while the solid line shows oxygen uptake in the presence of $2.5 \cdot 10^{-5}$ *M* thyroxine. Oxygen uptake was virtually linear in the presence of thyroxine. The control incubation on the other hand shows a drop in the oxidation rate after about 1 min, which is characteristic of succinate oxidation by these submitochondrial particles. All of the control rates of succinate oxidation reported in this paper were taken from the initial linear portion of the oxygen electrode traces.

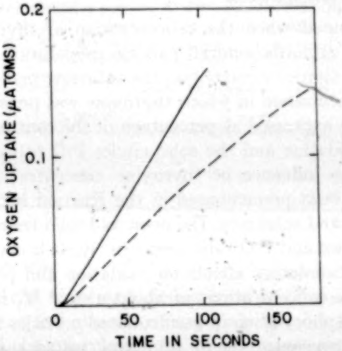

Fig. 1. Time course of succinate oxidation by submitochondrial particles, in the presence and absence of thyroxine. Dotted line, control; solid line, incubation in the presence of $2.5 \cdot 10^{-5}$ *M* thyroxine. For other conditions, see Table I.

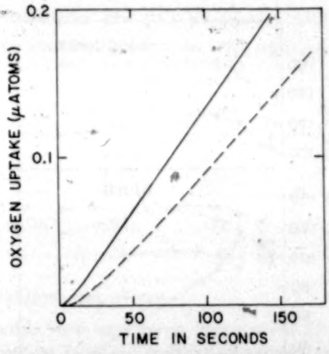

Fig. 2. Time course of DPNH oxidation by submitochondrial particles, in the presence and absence of thyroxine. Dotted line, control; solid line, incubation in the presence of $2.5 \cdot 10^{-5}$ *M* thyroxine. For other conditions, see Table I.

In Fig. 2 oxygen uptake curves are shown for the oxidation of DPNH by submitochondrial particles. Once again oxygen uptake in the presence of thyroxine is practically linear. The initial portion of the control curve is nonlinear, but after about 30 sec has elapsed a linear rate was established. The linear portion of the oxygen uptake traces were used to estimate the rates of DPNH oxidation reported in this paper.

When the time course of phosphorylation was studied with submitochondrial particles using either substrate the P/O ratios were found to remain approximately constant for time intervals up to about 3 min. Longer time intervals resulted in diminished P/O ratios both with and without thyroxine when succinate was the substrate.

Influence of thyroxine concentration

The results obtained when the concentration of thyroxine was varied using aliquots of the same submitochondrial particle preparation are shown in Fig. 3. In the upper part of the figure succinate was the substrate and in the lower part DPNH was used. For each incubation in which thyroxine was present the rate of oxidation and the P/O ratio are expressed as percentage of the control value. The open circles represent rates of oxidation and the solid circles P/O ratios. The lower part of the figure also shows the influence of thyroxine concentration on submitochondrial particles which have been preincubated in the reaction medium for 2 min prior to addition of thyroxine and substrate. The open and solid triangles give the percentage of control oxidative rate and P/O ratio respectively. It is evident for both substrates that the maximum stimulatory effects on oxidation and phosphorylation were observed with thyroxine concentrations of about $1 \cdot 10^{-5}$ *M*. In order to demonstrate the uncoupling of phosphorylation in preincubated particles 2 to $5 \cdot 10^{-5}$ *M* thyroxine had to be used. No concentration of thyroxine tested caused an increase in the oxidative rate of the preincubated particles in which uncoupling of phosphorylation occurred.

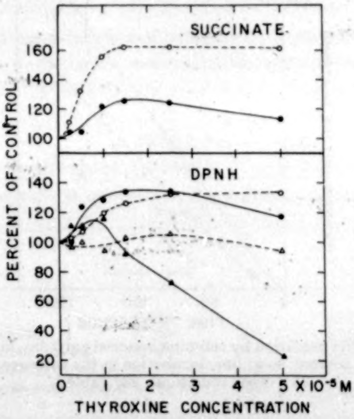

Fig. 3. The influence of various concentrations of thyroxine on oxidation and phosphorylation in submitochondrial particles. ○ – oxidation; ● – phosphorylation; △ – oxidation after 2-min preincubation; ▲ – phosphorylation after 2-min preincubation. All points were obtained by taking the percentage of the control value in the absence of thyroxine. 5 µmoles succinate or 2.5 µmoles DPNH as substrate; 0.07 mg protein nitrogen; for other conditions, see Table I.

Influence of thyroxine on ATPase and [^{32}P]ATP exchange

As the data in Table IV indicate, the improvement in P/O ratio in the presence of thyroxine was not accompanied by any comparable changes in the rate of [^{32}P]ATP exchange or in the ATPase activity of the submitochondrial particles.

TABLE IV

THE INFLUENCE OF THYROXINE ON ATPASE AND [^{32}P]ATP EXCHANGE

Conditions: for oxidative phosphorylation: 5 µmoles succinate as substrate, other conditions as in Table I; for ATPase: 5 µmoles ATP, 5 µmoles ADP, 5 µmoles PO_4, pH 7.0, 10 µmoles $MgCl_2$; for [^{32}P]ATP exchange: 5 µmoles ATP, 5 µmoles ADP, 10 µmoles $MgCl_2$, 20 µmoles PO_4, pH 7.0, $1 \cdot 10^6$ counts/min ^{32}P; 0.15 mg protein nitrogen; 1.9 ml total volume; 28°.

Thyroxine present (final concn.)	*Phosphorylation P/O ratio*		*ATPase (µmoles ATP split/min)*		*[^{32}P]ATP exchange (µmoles P exchanged/min)*	
		% control		*% control*		*% control*
0	0.58	—	0.60	—	0.096	—
$5 \cdot 10^{-5}$ *M*	0.75	129	0.62	103	0.100	104
$1 \cdot 10^{-4}$ *M*	0.73	126	0.63	105	0.105	109

Influence of DNP and thyroxine

Table V gives the results of an experiment in which the influence of thyroxine was studied in the presence and absence of 2,4-dinitrophenol. It is clear from these data that the blocking of phosphorylation by dinitrophenol did not alter the stimulation of oxidative rate by thyroxine.

TABLE V

THE INFLUENCE OF THYROXINE AND DNP ON SUBMITOCHONDRIAL PARTICLES

Conditions: 5 µmoles succinate as substrate, other conditions as in Table I.

Additions	*Oxygen uptake*		*P/O ratio*	
	(µatoms O/min)	*% control*		*% control*
None	0.209	—	0.64	—
$2.5 \cdot 10^{-5}$ *M* Thyroxine	0.261	125	0.78	122
$1 \cdot 10^{-4}$ *M* DNP	0.222	106	0.06	9
$2.5 \cdot 10^{-5}$ *M* Thyroxine and $1 \cdot 10^{-4}$ *M* DNP	0.279	133	0.07	11

Statistical significance of the thyroxine effects

Although it was possible with any given preparation of submitochondrial particles to obtain good agreement between replicate determinations, it is evident from the data presented above that there was considerable variability in the influence of thyroxine from one preparation to another. Consequently it was essential to determine whether the increases in oxidative rate and P/O ratio were statistically significant. Table VI shows the mean percentage of the control rate of oxidation and P/O ratio found in the presence of thyroxine for 10 different preparations where succinate was the substrate and 9 where DPNH was the substrate. For each preparation the percentage of the control value was calculated and these percentages were used to obtain the means and standard errors of the means shown in Table VI. The control values in the table are the means of the controls used to calculate the percentage in each case. For both substrates the mean percentage of control rates of oxidation and of control P/O ratios are significantly different from 100.

TABLE VI

STATISTICAL SIGNIFICANCE OF THYROXINE EFFECTS ON SUBMITOCHONDRIAL PARTICLES

The results in the presence of thyroxine are expressed as a percentage of the control values without thyroxine. In each case the mean and the standard error is given. The number of observations is shown in parentheses. The conditions of the incubations are given in Table I.

Substrate	*Oxygen uptake*		*P/O ratio*	
	Control (µatoms/min)	*Percentage of control with thyroxine present*	*Control*	*Percentage of control with thyroxine present*
Succinate	0.133 ± 0.012 (10)	144.2 ± 8.5 (10) [$p < 0.001$]	0.53 ± 0.04 (10)	131.2 ± 7.8 (10) [$0.001 < p < 0.005$]
DPNH	0.318 ± 0.028 (9)	119.7 ± 2.8 (9) [$p < 0.001$]	0.58 ± 0.04 (9)	113.2 ± 3.9 (9) [$0.005 < p < 0.01$]

DISCUSSION

The data presented above indicate three effects of thyroxine on submitochondrial particles. First, thyroxine increased the initial rate of oxidation of succinate and of DPNH. Secondly, the same concentrations of the hormone produced an improvement in the P/O ratio obtained with either substrate, indicating that the rate of phosphorylation was increased more than the rate of oxidation. The third effect of thyroxine, which was produced only at higher concentrations of the hormone, was the uncoupling of phosphorylation from the oxidation of either substrate. This uncoupling did not result in an increased oxidative rate.

The fact that the first two effects normally accompany one another raises the question of whether they are separate effects or different manifestations of the same action of thyroxine. Certainly, in these experiments the rate of oxidation does not appear to be limited by the rate of the terminal steps of the phosphorylation process, since thyroxine increased both the rate of phosphorylation and the rate of oxidation without increasing the rate of the [^{32}P]ATP exchange reaction. If thyroxine were acting to increase the rate of one of the reactions coupling the electron transport chain to the terminal steps of the phosphorylation process, one would expect, under circumstances in which these coupling reactions were rate-limiting for the electron transport process, that thyroxine would stimulate both the rate of oxidation and the P/O ratio. Such an action of thyroxine could be a result of the loss of thyroxine or of some substance replaceable by thyroxine from the phosphorylating mechanism during preparation of the submitochondrial particles. Previous reports from this laboratory[9,10] have presented evidence indicating that any substance which influenced the rate of phosphorylation without influencing either the ATPase activity or the rate of the [^{32}P]ATP exchange reaction would be likely to act on some stage of the phosphorylation mechanism prior to the involvement of inorganic phosphate. Therefore, the data in Table IV together with the fact that DNP does not alter the influence of thyroxine on oxidative rate, make it probable that any common site of thyroxine action on oxidation and phosphorylation would fall between the electron transport chain and the point at which inorganic phosphate is involved, and prior to the point of DNP inhibition of phosphorylation.

The above hypothesis would predict that intact mitochondria contain a sufficient quantity of coupling components to give optimal rates of oxidation and phosphorylation without added thyroxine, and this was observed. However, when mitochondria were washed in hypotonic phosphate the rates of oxidation and the P/O ratios were reduced to about one-half of those obtained with mitochondria in isotonic sucrose, and stimulation of oxidation and P/O ratio by thyroxine could readily be demonstrated. Once again, a decline in coupled phosphorylation made it possible to show improvement in rate of oxidation and P/O ratio in the presence of thyroxine. Whether such stimulation could account for the actions of the thyroid hormones *in vivo* would appear to depend on the extent to which the intramitochondrial level of thyroxine or of the component replaceable by thyroxine was influenced by the circulating level of the thyroid hormones.

Any possible stimulatory effect of thyroxine on the reactions responsible for coupling phosphorylation to the electron transport chain could not easily explain the uncoupling effects of higher concentrations of the hormone with intact mitochondria or submitochondrial particles. Before these effects can be understood more must be known about the changes that occur during the preincubation period which is an essential prelude to the uncoupling.

The effects of thyroxine on intact mitochondria reported in this paper are substantially in agreement with the findings of others[2-4, 11]. The results obtained with submitochondrial particles appear contradictory to the effects of thyroxine on preparations of this type in the hands of other investigators. However, the differences may be partly due to differences in the experimental methods employed. TAPLEY AND COOPER[3] have shown that thyroxine failed to influence oxidative phosphorylation in submitochondrial particles obtained by digitonin extraction of mitochondria. However, their incubations were of 20-min duration, and they did not make use of any method capable of giving them initial rates of oxygen uptake.

PARK *et al.*[12] have reported that thyroxine will uncouple phosphorylation from oxidation in sonic extracts of mitochondria without the necessity for any preincubation period. They obtained no stimulation of oxidation or phosphorylation. The differences between their results and those reported here may be partly due to the longer incubation times and the failure to measure initial oxidative rates, but more probably they result from the fact that these investigators did not centrifuge the submitochondrial particles from the supernatant soluble protein in the sonic extracts. During the present study it was found that additions of soluble protein intensified the uncoupling action of thyroxine and often obscured the stimulatory effects of the hormone.

WOLFF AND BALL[13] have shown that the stimulation of succinate oxidation by thyroxine in rat heart homogenates was due to an inhibition of malic dehydrogenase. It is clear that these investigators were dealing with a type of effect different from the stimulation of succinate and DPNH oxidation reported here, since they found no change in the initial rate of succinate oxidation. WOLFF AND WOLFF[14] have extended the above observations with studies on the inhibition of several other dehydrogenases by thyroxine and its analogues. However, it is difficult, as they point out, to relate this type of effect to the *in vivo* stimulation of oxidative rate by thyroxine.

In fact, apart from the possible connection between the uncoupling of oxidative phosphorylation and the effects of thyroxine in thyrotoxicosis[4, 11], it seems unlikely that any purely inhibitory effects of thyroxine could explain its normal physiological actions, even if an inhibition of phosphorylation resulted in an increase in oxidative rate. The results presented in this paper are compatible with the notion that thyroxine acts by direct stimulation of the terminal oxidative processes of the cell.

ACKNOWLEDGEMENT

The author wishes to express his sincere thanks to Dr. W. W. Kielley for much advice and encouragement during the course of this work.

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Biochim. Biophys. Acta, 37 (1960) 327–336

ROTATORY BEHAVIOR OF PROTEIN DENATURATION

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(Received October 28th, 1958)

SUMMARY

Reversible and irreversible denaturation processes were examined by rotatory dispersion. Samples of bovine serum albumin that were submitted to reversible denaturation by urea attained their original conformation after removal of the denaturing agent. In the irreversible denaturation all proteins were completely unfolded or slightly associated in the β-form in the presence of denaturant. But after the removal of the denaturant striking formation of the β-form was observed. The reversibility of the denaturation seems to depend on the nature of the solvent.

Biochim. Biophys. Acta, 37 (1960) 336–341

INTRODUCTION

It is well known that optical rotatory power is very sensitive to changes in molecular configuration. When proteins are subjected to denaturation it is believed that the polypeptide chains are uncoiled, without precipitating from solution, and that this change of configuration is reflected in the change of optical rotatory power. For example, JIRGENSONS[1], GOLUB AND PICKETT[2] and SCHELLMAN[3] have recently studied the changes in the optical rotations of many proteins that were denatured in various ways and have found out that $(\alpha)_{\mathrm{D}}$ increases in absolute value from 20 to 70°.

It is important to note, however, that for a given protein the value of $(\alpha)_{\mathrm{D}}$ depends to some extent on the solvent as well as the configuration. Through the work of DOTY's group[4] the poly-γ-benzyl glutamate molecule was shown to have the same unfolded configuration in dichloroacetic acid and trifluoroacetic acid. However, $(\alpha)_{\mathrm{D}}$ in the former solvent was $-14°$, but in the latter solvent it was as low as $-41°$. This difference can only be explained by the natures of the solvents.

Since $(\alpha)_{\mathrm{D}}$ is very sensitive to the configuration of the chain, the presence of the β-form or the α-helix of the other screw direction should also have some effect upon the rotatory power. YANG AND DOTY[4] have discovered that poly-γ-benzyl glutamate of very low molecular weight showed $(\alpha)_{\mathrm{D}}$ of 10° at higher concentration and $-14°$ at lower concentration, while infrared spectrum results[5] have proved the configuration to be β-form in the concentrated solution and random coil in the dilute solution. This indicates that β-form makes a positive contribution to the rotatory power.

Considerable ambiguity can arise in interpreting the results of the optical rotation if helices of two directions coexist. That this may occasionally occur was indicated by the following observations. The $(\alpha)_{\mathrm{D}}$ values of almost all proteins become more positive upon transferring them from water to 2-chloroethanol. This is most probably due to an increase of helical content brought about by this process. Thus, the $(\alpha)_{\mathrm{D}}$ value of ovalbumin goes from $-31°$ to $+11°$ and that of bovine serum albumin from $-63°$ to $-24°$. Insulin, however, behaves differently. Its $(\alpha)_{\mathrm{D}}$ value remains constant at $-36°$ in both solvents, while a very high helical content is found in 2-chloroethanol by an infrared spectroscopic method[6]. The inconsistency is most simply explained by assuming the coexistence of some left-handed helix[*]. Since the left-handed helix is known to make a negative contribution to $(\alpha)_{\mathrm{D}}$ an increase in right-handed helix would be masked by the increase in left-handed helix, in the rotatory behaviour of a mixture.

Summarizing the above results, we can say that the right-handed helix and the β-form make positive contributions, while the left-handed helix and the random coil make negative contributions to $(\alpha)_{\mathrm{D}}$ values. As a consequence it is quite difficult to reach an explicit conclusion concerning the conformation of the polypeptide chain only from $(\alpha)_{\mathrm{D}}$ results.

To decrease the ambiguousness, the optical rotatory dispersion, rather than the optical rotation at one wavelength should be employed. Although the theory of optical rotatory power as related to molecular structure is still not well established, the dispersion data for protein solution can be expressed by the following empirical equation[6]

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[*] There is no direct evidence to prove that the α-helix of the typical polypeptides or proteins is of the right-handed type. But in order to make the following discussion simple we should like to assume it to be so. In any event, we can always tell one direction of helix from the other through rotatory dispersion results as described under RESULTS.

$$(\alpha) = \frac{100}{M}\frac{n^2+2}{3}\left[\frac{a_0^R\,\lambda_0^2}{\lambda^2-\lambda_0^2}+\frac{a_0^s\,\lambda_0^2}{\lambda^2-\lambda_0^2}+\frac{b_0^s\,\lambda_0^4}{(\lambda^2-\lambda_0)^2}\right] \qquad (1)$$

Here, a_0^R refers to the intrinsic residue rotation. M is the mean residue weight and n is the refractive index of the solvent. The second and the third terms in the bracket come from the helical or any conformations other than the random or disordered one and the magnitude of a_0^s and b_0^s should be proportional to the content of such a conformation. When the protein is denatured and the polypeptide chain is uncoiled, the second and the third terms vanish so that the dispersion data can be expressed only by the first term. a_0^s and b_0^s for the pure right-handed helix are estimated to be +680 and —600, respectively, assuming $\lambda_0 = 212$ mμ. Since the positive contribution of the second term is more substantial at longer wavelengths than the negative contribution of the third term, $(\alpha)_D$ becomes less positive as the helical content decreases.

The dispersion data of the β-form obtained by YANG AND DOTY[4] can also be expressed perfectly by eqn. (1). At present, we cannot estimate the magnitudes of a_0^s and b_0^s for the pure β-form correctly, because the determination of the amount of β-form in the solution has not been established. But it is quite evident from the dispersion data of YANG AND DOTY that both a_0^s and b_0^s should be positive. It is true that positive b_0^s can be expected for the left-handed helix too, but the left-handed helix has negative a_0^s and we can tell the β-form from the left-handed helix by this means.

Summarizing the above discussion, we can say

(1) If a_0^s is positive and b_0^s is negative the molecule should contain a right-handed helix.

(2) If a_0^s is negative and b_0^s is positive the molecule should contain a left-handed helix.

(3) If both a_0^s and b_0^s are positive the β-form should be dominant.

(4) If a_0^s and b_0^s are zero the molecule should be completely uncoiled.

Thus we have a way of identifying the major or dominating contribution to secondary structure by the method of rotatory dispersion.

In the course of much previous work it became apparent that protein molecules are unfolded when denatured. But the configuration taken by proteins after the denaturing condition is removed has not been widely examined. Much evidence has been obtained to show that the denatured protein molecule has the β-form in the solid film, while there are good reasons to believe that the denatured protein molecule is uncoiled and has a random coil configuration in the solution. Since these two possible forms of denatured molecules are both found to exist in the solution of several polypeptides[6], it is quite reasonable that denatured protein would take either the β-configuration or the random coil configuration, depending on the condition of denaturation. The work reported below was undertaken in order to see what information rotatory dispersion could furnish on the configuration of some typical proteins in the native and denatured forms.

EXPERIMENTAL

***Polarimeter*.** An especially constructed spectrophotometric polarimeter was used. The light emitted by a tungsten lamp was monochromatized by a Hilger monochrometer, passed into a Goerz (German) polarimeter and was detected by a 931A (RCA) photo-multiplier tube. The position of complete extinction was determined by a so-called symmetrical angles method. Two analyzer positions, defined by the readings θ_{0a} and θ_{0b} on a graduated scale in the absence of the optically active substance and by θ_{1a} and θ_{1b} in the presence of the substance, are determined for which the galvanometer deflections are equal. Then optical rotation α can be obtained as

$$\alpha = \theta_{E1} - \theta_{E0} = (\theta_{1b} + \theta_{1a})/2 - (\theta_{0b} + \theta_{0a})/2 \qquad (2)$$

θ_E being the interpolated reading which would correspond to extinction. α can be reduced to specific rotation (α) through the equation

$$(\alpha) = \frac{100}{C}\,\frac{\alpha}{l} \qquad (3)$$

where C is the concentration expressed in g/100 ml, and l is the length of polarimeter in dm.

Spectral determinations. Infrared spectra were run in a Model 21 Perkin Elmer double-beam spectrometer using a sodium chloride prism. Films for these determinations were cast on mica plates and dried at room temperature in a desiccator.

Samples. Bovine serum albumin was obtained from Armour Company (Lot number N 120826). Ovalbumin was a sample prepared by Dr. KATZ. The concentrations of these protein solutions were determined by the Kjeldahl nitrogen method.

RESULTS

Bovine serum albumin

Rotatory dispersion data under different conditions are shown in Fig. 1. In the figure, $(\alpha)(\lambda^2 - \lambda_0^2)$ is plotted against $1/(\lambda^2 - \lambda_0^2)$. If the dispersion data can be expressed by eqn. (1), this plot should give a straight line. The slope of the line should be equal to $100\,(n^2 + 2)\,b_0^s\,\lambda_0^4/3\,M$ and the intercept is equal to $100(n^2 + 2)$ $(a_0^s + a_0^R)\,\lambda_0^2/3\,M$.

As is obvious from the figure, the dispersion data of the solution in 8 M urea gave $b_0^s = 0$ and thus, the intercept gave directly the value of a_0^R to be —620. $(\alpha)_D$ in this solution is —98°. The original sample, which gave $(\alpha)_D$ to be —58.1° furnished the following data: $b_0^s = -170$ and $a_0^s = +291$.

When this sample was exposed to alkaline (pH 12) solution $(\alpha)_D$ went down to —98.7°, but b_0^s changed its sign to positive suggesting the presence of a slight amount of β-form. This solution was then brought to pH 6 again. As $(\alpha)_D$ went back to —53.2° the configuration were also supposed to be recovered. But the dispersion in Fig. 1 showed the extraordinary result: b_0^s was about +234 and a_0^s was about +250. These positive a_0^s and b_0^s prove exclusively that the molecule is changed to the β-form. The process of exposing the protein to alkaline solution and bringing it back to neutral pH appears to be quite favorable for β-form formation. The existence of β-form could be confirmed by examining the infrared spectra of the precipitate obtained after dialysing out the salt. The existence of a sharp and remarkable 1625 cm^{-1} CO-stretching band proved that this was the β-form. Thus the alkaline denaturation of protein is irreversible and one might be able to assume that any irreversible denaturation is accompanied by β-form formation. To prove this assumption one has to check irreversible denaturation of a type other than alkaline denaturation. The typical example of irreversible denaturation is thought to be heat denaturation. Bovine serum albumin was denatured according to STELBERG's method[8] at 100° for 10 min. 60% *n*-propyl alcohol was used as the solvent to prevent precipitation. $(\alpha)_D$ was $-61.0°$, almost equal to that of the native one. The dispersion of the solution thus obtained is plotted in Fig. 1. Apparently it has positive a_0^s and b_0^s and thus the existence of the β-form is proved.

This protein was also exposed to the action of 8 *M* urea. The molecule was completely unfolded as is evident from the dispersion data which is shown in Fig. 1. The solution was then put in a dialysis bag and was dialysed against water for 48 h An ample amount of protein was precipitated and removed by centrifugation at a speed of 20,000 rev./min. The resultant solution gave $(\alpha)_D = -58.2°$, $a_0^s = 289$ and $b_0^s = -315$ corresponding to 44 to 55% of helical content. This result means that the protein which was still remaining in the solution after the dialysis was essentially converted into the original configuration again. The higher content of the helix observed in the resultant solution in comparison with the original one means that an ample amount of the original sample was denatured into the β-form, which resulted in such a low b_0^s. As this denatured sample was removed from the solution through the dialysis process the helical content of the final solution was raised.

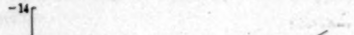

Fig. 1. Rotatory behavior of bovine serum albumin.

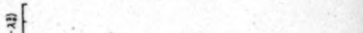

Fig. 2. Rotatory dispersion of ovalbumin. (1) natural ovalbumin (2) denatured ovalbumin in 8 *M* urea.

Ovalbumin

This protein is very unstable and easily denatured. If once this is denatured by 8 *M* urea and the urea then dialysed off from the solution the protein precipitates perfectly. This means that even urea denaturation is of the irreversible type. Actually the dispersion of ovalbumin in 8 *M* urea, which is shown in Fig. 2, shows b_0^s of $+270$ indicating that β-form association occurred in even such a concentrated urea solution as 8 *M*, though the $(\alpha)_D$ value of $-78°$ would indicate that the random coil is predominant in this solution. The native sample gave $(\alpha)_D$ of $-32.3°$ and dispersion data of Fig. 2, which corresponded to a b_0^s value of -200.

DISCUSSION

It is interesting to note that the protein is transformed into helical form or β-form upon reversal of denaturation, depending on the method of denaturation. Such a competition between helix and β-form can be observed in the coil-helix transition of synthetic polypeptides[9,10]. A copolymer of alanine and benzylglutamate (3:7) has random coil form in trifluoro-acetic acid and the helical form in ethylene dichloride. Solutions of equal copolymer concentrations in these two solvents were mixed in varying proportions and the optical rotation was determined. A small increase in optical rotation was observed upon the first addition of trifluoroacetic acid solution, until a sharp change occurred in the vicinity of 85 volume % ethylene dichloride. On examining the infrared spectrum of the solution, we found a remarkable amount of β-form present besides the helical form. At lower concentrations of ethylene dichloride the properties were those of the pure trifluoroacetic acid. The chain is completely unfolded. This means that just in the region of coil-helix transition competition takes place between those two forms. Since this transition is caused only by changing the composition of the solvent, the nature of the solvent seems to rule the choice of the configuration of the chain. The reversibility of protein denaturation may also depend on the nature of the solvent.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. M. KAWANO of the Research Laboratory of the Railway Company of Japan for help in the work of infrared spectrum and to Professor DOTY and Mrs. ELIZABETH KLEMPERER at Harvard University for their valuable advice and discussions.

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Short Communications

Theoretical considerations concerning the reaction of molecular oxygen and cytochrome oxidase

Studies on the reaction mechanism between reduced cytochrome oxidase (a_3)* and molecular oxygen have taxed biochemists for more than 25 years. Several hypotheses[1–7] have appeared, but none seems entirely satisfactory. All of these hypotheses have avoided the use of higher molecularity for the following reaction:

$$4a_3^{+2} + O_2 + 4H^+ \rightarrow 4a_3^{+3} + 2H_2O \tag{1}$$

In view of the extreme efficiency of the terminal oxidation, it is, indeed, most unlikely that the reaction would proceed as written.

In any attempt to elucidate the mechanism of enzymic reactions by kinetic treatment, one should consider the enzyme as a reacting entity, as indicated in the concept of the enzyme–substrate complex as advanced by HENRI, and MICHAELIS AND MENTON. On this basis, a consideration of the involvement of iron in high valencies like those in peroxidase and catalase[8,9] coupled with mechanisms involving free radicals, leads to our suggestion that the oxidation of reduced cytochrome oxidase may proceed according to the following reaction sequence:

$$a_3^{+2} + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} a_3^{+2}O_2 \tag{2}$$

$$a_3^{+2}O_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} a_3^{+3} + O_2^- \tag{3}$$

$$O_2^- + H^+ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} HO_2^{\cdot} \tag{4}$$

$$a_3^{+2} + HO_2^{\cdot} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} a_3^{+3} + HO_2^- = (a_3OOH)^{+2} \tag{5}$$

$$(a_3OOH)^{+2} + a_3^{+2} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} (a_3O)^{+2} + a_3^{+3} + OH^- \tag{6}$$

$$(a_3O)^{+2} + H^+ + a_3^{+2} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} a_3^{+3} + OH^- + a_3^{+3} \tag{7}$$

$$2OH^- + 2H^+ \rightarrow 2H_2O \tag{8}$$

The overall reaction is:

$$4a_3^{+2} + O_2 + 4H^+ \rightleftharpoons 4a_3^{+3} + 2H_2O \tag{9}$$

The rate of the oxidation of reduced cytochrome oxidase can then be derived as follows:

$$\frac{-\,d(a_3^{+2})}{dt} = k_1(a_3^{+2})\,(O_2) + k_4(a_3^{+2})\,(HO_2^{\cdot}) + k_5(a_3^{+2})\,((a_3OOH)^{+2}) + k_6(a_3^{+2})\,((a_3O)^{+2})\,(H^+) \tag{10}$$

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* In this paper, cytochrome oxidase is considered identical with cytochrome a_3, unless otherwise indicated.

when K_{-6}, K_{-5}, K_{-4}, etc. are negligible, *i.e.* the dissociation of intermediates to original components are much less than their formation; then also:

$$\frac{\mathrm{d}(a_3{}^{+2}O_2)}{\mathrm{d}t} = k_1(a^{+}{}_3{}^{2})\,(O_2) - k_2(a_3{}^{+2}O_2) \tag{11}$$

$$\frac{\mathrm{d}(O_2{}^{-})}{\mathrm{d}t} = k_2(a_3{}^{+2}O_2) - k_3(O_2{}^{-})\,H^{+} \tag{12}$$

$$\frac{\mathrm{d}(HO_2\cdot)}{\mathrm{d}t} = k_3(O_2{}^{-})\,(H^{+}) - k_4(a_3{}^{+2})\,(HO_2\cdot) \tag{13}$$

$$\frac{\mathrm{d}((a_3OOH)^{+2})}{\mathrm{d}t} = k_4(a_3{}^{+2})\,(HO_2\cdot) - k_5((a_3OOH)^{+2})\,(a_3{}^{+2}) \tag{14}$$

$$-\frac{\mathrm{d}((a_3O)^{+2})}{\mathrm{d}t} = k_5((a_3OOH)^{+2})\,(a_3{}^{+2}) - k_6((a_3O)^{+2})\,(a_3{}^{+2})\,(H^{+}) \tag{15}$$

and at steady state:

$$\begin{aligned} k_1(a_3{}^{+2})\,(O_2) &= k_4(a_3{}^{+3})\,(HO_2\cdot) = k_5(a_3{}^{+2})\,((a_3OOH)^{+2}) \\ &= k_6(a_3{}^{+2})\,((a_3O)^{+2})\,(H^{+}) \end{aligned} \tag{16}$$

The final form thus becomes:

$$-\frac{\mathrm{d}(a_3{}^{+2})}{\mathrm{d}t} = 4k_1(a_3{}^{+2})\,(O_2) \tag{17}$$

Reactions (3) and (4), in fact, can be considered as:

$$H^{+} + a_3{}^{+2}O_2 \rightleftharpoons a_3{}^{+3} + HO_2\cdot \tag{18}$$

It is true that k_2 may be small, *i.e.* $k_3 \gg k_2$, and reaction (3) is slightly endergonic. But reaction (4) is exergonic (E_0 is about 0.5 V) and its activation energy approaches zero[10]. Since $O_2{}^{-}$ is weakly acidic, $O_2{}^{-}$ would combine with a proton to form $HO_2\cdot$ instantaneously, just like the reaction of a proton and OH^{-} to yield water. Thus the low rate constant of reaction (3) would be compensated.

From the formulation, the rate of the oxidation is first order with respect to cytochrome a_3 and oxygen. The final form of the kinetic expression bears some resemblance to that of LUVALLE AND GODDARD[7]. However, according to these authors, the rate of oxidation is dependent on cytochrome oxidase and cytochrome *c*, and the concentration of oxygen does not appear in the overall rate equation. Also according to them oxygen reacts only with the a_3–c binary complex. Moreover, their intermediate complexes, like $a_3O_2{}^{-}$, $a_3\cdot O_2H$, and a_3OH, would undergo spontaneous reaction to inactivate the oxidase unless their lives are extremely short. If so, cytochrome oxidase should be more stable in the presence of cytochrome *c* than its absence in order to keep the lives of free radical-enzyme complex short. This is not consistent with the known properties of the oxidase[11]. In contrast to their derivation, the proposed mechanism does not meet with the objection of spontaneous inactivation of the oxidase by free radicals. Moreover, the mechanism is in line with known properties of cytochrome oxidase and conforms to the modern concept of enzyme chemistry. In other words, reduced cytochrome oxidase is a reactant and is oxidized by molecular oxygen, another reactant, in the oxidation. Treatment of this step of oxidation with respect to the mechanism and kinetics does not need to include cytochrome *c*. This is also an essential point which differs from the hypothesis suggested by SLATER[4].

Recently TAKEMORI *et al.*[12] have reported that c^{+2}, but not hydroquinone, is required for the oxidation of cytochrome oxidase. Sekuzu *et al.*[13] and Minnaert[14] have found that the oxidized a_3 formed with O_2 is different from that with ferricyanide. These findings do not militate against the proposed mechanism but only illustrate the specificity of the reactions. In fact, the results by Takemori *et al.* can be explained on the basis that the reduced oxidase formed with hydroquinone or *p*-phenylenediamine is not the same as that formed with reduced cytochrome *c*.

Several moot questions could be resolved by experiments with pure reduced cytochrome oxidase. The present difficulty lies in the preparation of the reduced oxidase in the "native" form but without contamination.

This work was supported by the National Science Foundation and is published with the approval of the Monographs Publications Committee, Research Paper No. 364, School of Science, Department of Chemistry.

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Received April 6th, 1959

Biochim. Biophys. Acta, 37 (1960) 342–344

Bond cleavage in enzymic hydrolysis of nucleoside triphosphate to nucleoside monophosphate and inorganic pyrophosphate

The hydrolysis of ATP to form inorganic orthophosphate and ADP catalyzed by several different enzymes, namely myosin[1], a soluble Mg^{++}-activated ATPase of muscle[2] and potato apyrase[3], occurs in every case with cleavage of the bond between the terminal P and O of ATP. These reactions have been described as displacement reactions with a nucleophilic attack on the terminal P and the enzymes may be considered phosphoryl-transferring enzymes with H_2O as the acceptor analogous in mechanism to the kinase reactions which have other acceptors.

An ever increasing number of reactions involving the cleavage of the nucleotidyl group of nucleoside triphosphates with the concomitant formation of inorganic pyrophosphate are being found. Boyer *et al.*[4] have demonstrated a cleavage of the bond between the P of the adenyl moiety and O in the reaction of ATP with acetate

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Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate, ATP, adenosine triphosphate; d-CTP, deoxycytosine triphosphate; d-CMP, deoxycytosine monophosphate; CoA, coenzyme A.

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and CoA to form acetyl-CoA, AMP and inorganic pyrophosphate indicating that the enzyme catalyzes a transfer of the adenyl group. It may well be that all members of this class of reactions have the same type of bond cleavage. The present investigation was undertaken to determine whether the enzymic hydrolysis of nucleoside triphosphates to form nucleotides and inorganic pyrophosphate occurred by cleavage of the bond between the P of the nucleotidyl moiety and O or between the middle P and O. Two reactions have been studied, the hydrolysis of ATP by a cobra venom (*naia naia*) preparation[5] and the hydrolysis of d-CTP by an enzyme from T2-infected *Escherichia coli*[6].

The hydrolysis of ATP by cobra venom was carried out in ^{18}O-enriched water. In the first experiment, 25 µmoles ATP were incubated in 0.05 *M* tris(hydroxymethyl) aminomethane, pH 8.5, with 3.9 mg dried venom in a total vol. of 5 ml for 30 min at 30°; 0.1 ml of the reaction mixture was removed to analyze the medium for ^{18}O. The reaction was stopped with $HClO_4$, final concn. 6 %. After removal of the unhydrolyzed ATP with norite from the $HClO_4$ supernatant, analysis for inorganic orthophosphate and hydrolyzable phosphate showed that 11.6 µmoles orthophosphate and 9.2 µmoles pyrophosphate had been formed. In a second experiment, 30 µmoles ATP were similarly incubated with 5.7 mg venom for 1 h at 30°. After similar treatment of the reaction mixture, it was found that 26.3 µmoles orthophosphate and 13.3 µmoles pyrophosphate had been formed. The orthophosphate results from the combined action of 5′ nucleotidase and inorganic pyrophosphatase present in cobra venom[5]. The manifestation of the various enzymic activities in the venom depends on the concentration of venom used because of the Mg content of the venom and the varying sensitivities of the different enzymic activities to the Mg^{++} concentration. The orthophosphate and pyrophosphate produced in the reaction were separated chromatographically on a Dowex 1 column as described previously[7]. The pyrophosphate fraction was hydrolyzed in normal H_2O and the orthophosphate formed was precipitated as the Ba salt. The phosphate and water of the medium were analyzed for ^{18}O as described previously[8]. The absence of ^{18}O in pyrophosphate shown in Table I indicates that cleavage of ATP occurred between the P of the adenyl moiety and O.

In Expt. 2 (Table I), the orthophosphate fraction (26.3 µmoles) isolated directly from the reaction mixture was also analyzed for ^{18}O. The maximum value of ^{18}O anticipated was equivalent to 1.5 atoms. This calculated value is based on the assumption that one-third of the total phosphate (52.9 µmoles) cleaved in the reaction, *i.e.* 17.6 µmoles, was orthophosphate derived from the adenyl phosphate by the sequential action of ATPase and 5′ nucleotidase and would contain 2 atoms O from

TABLE I

HYDROLYSIS OF NUCLEOSIDE TRIPHOSPHATES YIELDING INORGANIC PYROPHOSPHATE

		^{18}O concn. (atom % excess)			
Substrate	*Enzyme source*	H_2O	*Phosphate (from pyrophosphate)*		
			Observed	*Calculated*	
				XP-¦-O-PP	*XP-O-¦-PP*
(a) ATP	Snake venom (*naia naia*)	1.30	0.000	0	0.163
(b) ATP	Snake venom	1.22	0.000	0	0.153
(c) d-CTP	T2-infected *E. coli*	10.10	0.084	0	1.263

H_2O and the remainder of the orthophosphate fraction, 8.7 μmoles, was derived from the middle and terminal phosphate by the action of inorganic pyrophosphate and would contain ½ atom O from H_2O/orthophosphate. The observed value was 0.361 atom % excess ^{18}O, equivalent to 1.2 atoms.

The hydrolysis of d-CTP by an enzyme purified from T2-infected *E. coli* was also carried out in ^{18}O-enriched water. The final concns. in the 1.0-ml incubation mixture were d-CTP, 0.002 *M*; $MgCl_2$, 0.008 *M*; dimercaptoethanol, 0.01 *M*; glycine, pH 9.2, 0.04 *M*; and 0.2 unit[6] of enzyme. The mixture was incubated at 37° and a 30-μl sample was withdrawn for ^{18}O analysis of the medium. At the end of 1 h the reaction mixture was lyophilized to remove the $H_2{}^{18}O$. The residue was suspended in 2 ml cold 5 % trichloroacetic acid and treated with norite to remove the unreacted d-CTP and d-CMP which had been formed. The inorganic pyrophosphate in the supernatant and washings of the norite suspension was hydrolyzed. The orthophosphate formed was diluted with carrier phosphate and precipitated as the Ba salt and analyzed for ^{18}O. As shown in Table I, there was essentially no ^{18}O in the pyrophosphate formed. The small amount of ^{18}O in the product is undoubtedly due to contamination of the pyrophosphate with orthophosphate formed by pyrophosphatase which was an impurity in the enzyme preparation.

The results of these experiments indicate that the hydrolysis of nucleoside triphosphates by the two enzymes studied to yield nucleotide and inorganic pyrophosphate may be considered nucleotidyl transfers proceeding by attack on the P of the substituted terminal phosphate of the triphosphate rather than an attack on the middle P. The demonstration of this type of bond cleavage in hydrolyses of this type adds another piece of experimental evidence to the generalization that the middle P of triphosphates are resistant to attack and that reaction occurs most easily by attack on a terminal P.

The author wishes to thank Dr. A. KORNBERG and Mr. S. ZIMMERMAN for supplying the d-CTP and the enzyme d-CTPase and also wishes to acknowledge the technical assistance of Mr. S. KESSLINGER. This work was supported by a research grant of the National Science Foundation and carried out during the tenure of an Established Investigatorship of the American Heart Association.

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Received May 9th, 1959

Action of ribonuclease on a microsomal ribonucleoprotein

The microsomes of rat liver and the dense ribonucleoprotein granules which are a major component of this family of complex submicroscopic particles have formed the subject of numerous studies of which only a few can be mentioned[1-4]. The effect of pancreatic ribonuclease and other nucleases on uncombined ribonucleic acids has been studied much more frequently[5] than the enzymic degradation of ribonucleic acid that is linked to protein[2,6-8]. It appeared of some interest to carry out a few orienting studies on the enzymic degradation of the RNP particles from rat-liver microsomes. The effect of various bivalent cations on the action of the enzyme was also followed.

The RNP particles were isolated from the microsomes of rat-liver cytoplasm by the methods described previously, with the use of sodium deoxycholate[3]. The preparations were suspended in 0.3 *M* sucrose and used for the enzymic studies either immediately or after brief storage at 5°, but never for more than one night. Crystalline ribonuclease from ox pancreas was a commercial preparation (Armour). Magnesium and manganese were used as the chlorides, copper and zinc as the acetates.

The procedure for the assay of enzyme action followed a previous publication[9] with slight modifications. In order to prevent the spontaneous degradation of the unstable conjugated protein, it was necessary to perform the enzyme experiments at a low temperature for which reason relatively high enzyme concentrations were employed. To 1.5 ml of a RNP suspension (7–8 mg dry wt.) in sucrose, 0.2 ml 1 *M* sodium acetate, pH 6.0, and 0.1 ml of a ribonuclease solution (containing 0.5 mg enzyme) were added. The mixture was rapidly transferred to a small dialysis bag (Visking) and subjected to dialysis against 110 ml 0.1 *M* sodium acetate, pH 6.0, at 4–5°. Parallel control experiments, in which the enzyme was omitted, were carried out simultaneously. Portions of the dialysate were removed at definite intervals and analyzed for their absorption in the u.v. and for organic P. The rate of appearance of phosphorylated, u.v.-absorbing substances served as a measure of the hydrolysis of RNP.

In the experiments in which the effect of bivalent cations or of urea on the enzymic hydrolysis of RNP was investigated, these substances were added to the enzymic digestion mixtures at the beginning of the experiments. They were also present, in the same concentrations, in the buffer solution serving as the outside dialysis fluid.

The extreme instability of the ribonucleoprotein particles at higher temperatures necessitated the performance of the enzymic experiments at 4–5°. The RNP particles were found to be stable at 4° for at least 14 days, whereas at 25° about 40–50 % of the preparation decomposed after 4 h, as estimated from the amount of u.v.-absorbing material that remained non-sedimentable after centrifugation at 105,000 × *g* for 1 h. The course of the enzymic digestion of RNP and the inhibiting effect of Mg^{++}, down to a concentration of 10^{-3} *M*, are shown in Fig. 1. At a concentration of 10^{-4} *M* Mg^{++} inhibition was no longer noticeable. The inhibition by 0.01 *M* concentrations of Zn^{++} and Mn^{++} is illustrated in Fig. 2. Similar effects were observed with the same concentration of Cu^{++}.

For purposes of comparison, ribonucleic acid was prepared from the microsomal

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Abbreviation: RNP, ribonucleoprotein.

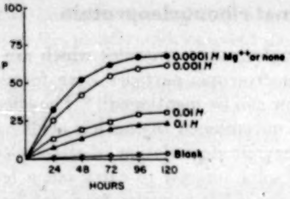

Fig. 1.

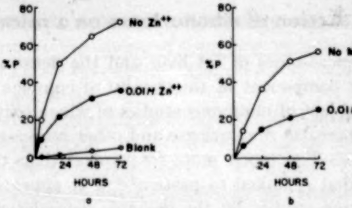

Fig. 2.

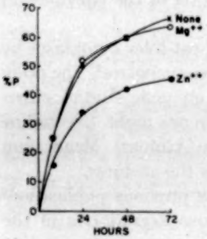

Fig. 3.

Fig. 1. Hydrolysis of the ribonucleoprotein particles by pancreatic ribonuclease in the presence of the indicated concentrations of $MgCl_2$. The composition of the digestion mixtures is given in the text. In the blank experiment enzyme and Mg^{++} were omitted. The ordinate indicates the percentage of ribonucleoprotein P rendered dialyzable at the time given on the abscissa.

Fig. 2. Enzymic hydrolysis of RNP in the presence of Zn^{++} or Mn^{++}. See Fig. 1 for other explanations.

Fig. 3. Enzymic hydrolysis of the ribonucleic acid from RNP in the presence or absence of Mg^{++} or Zn^{++} (both 0.01 *M*).

RNP granules of rat liver by extraction with hot NaCl, purified as described previously[10], and similarly treated with pancreatic ribonuclease in the presence and absence of Mg^{++} and Zn^{++}. The results shown in Fig. 3 demonstrate that Zn^{++} interfered with the full enzymic effect, whereas Mg^{++} did not, in agreement with previous observations[11-13].

It will thus be seen that of the bivalent ions tested all inhibited, to a certain extent, the enzymic attack on the ribonucleoprotein, whereas only zinc interfered with the enzymic degradation of the sodium ribonucleate itself. It may be assumed that the gradual dissociation of the ribonucleoprotein is among the prerequisites for enzymic splitting and that magnesium[14] and probably also manganese have a stabilizing effect on the conjugated protein. The inhibition by the heavy metals, on the other hand, may follow a different mechanism, *viz.*, through their being bound in some manner to the nucleic acid[15] and thus interfering with the enzymic attack.

Another observation may be adduced in favor of this view. It has recently been made probable[16] that hydrogen bonds are involved in the structure of ribonucleoproteins and that a complete dissociation of nucleic acid and protein can be effected by urea. Such action could have been expected to facilitate the hydrolysis of the liberated nucleic acid by ribonuclease. This is actually the case, as shown in Table I, with the initial rate of enzymic hydrolysis showing a definite increase in the presence of 4 *M* urea. It is interesting that very prolonged treatment (20 h or longer) with urea produced some breakage to dialyzable fragments even in the absence of the enzyme.

By far the largest part of the ribonucleic acid of rat-liver cytoplasm occurs in the form of nucleoproteins; only a small portion of the supernatant RNA is not combined with protein[17]. It may be assumed that the almost universal conjugation of ribonucleic acid to proteins is not without relation to the control of its enzymic degradation within the cell.

TABLE I

EFFECT OF $4\,M$ UREA ON THE ENZYMIC DEGRADATION OF RNP

	% of ribonucleoprotein P rendered dialyzable					
	Digestion time (min)					
	12	48	108	252	1200	2400
Urea	3	18	34	46	57	80
No urea	2	7	12	26	52	64
Control[*]	0	0	1	3	8	20

[*] In the control experiment RNP and urea, but no enzyme, were present.

We should like to acknowledge the assistance of Mr. C. N. GORDON. This work has been supported by research grants from the National Institutes of Health, Public Health Service, and the National Science Foundation.

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Received May 14th, 1959

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Biochim. Biophys. Acta, 37 (1960) 347–349

Degradation of DNA by the Ehrlich ascites tumor

Ehrlich ascites cells contain at least two enzymes capable of cleaving DNA. One of these is optimally active at pH 8.5, rapidly degrades heat-denatured DNA but is virtually inactive against native DNA. The other is optimally active at about pH 5 and is similar to DNase II of CUNNINGHAM AND LASKOWSKI[1]. They will be referred to as "alkaline" and "acid" enzymes, respectively.

Both enzymes are readily extracted from tumor cells by repeated freezing and

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Abbreviation: DNA, deoxyribonucleic acid.

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thawing in physiological saline. Approximately 50 % of the activity occurred in the supernatant fluid after centrifugation at 100,000 × g for 30 min. Treatment of particle-free extracts with $(NH_4)_2SO_4$ at pH 7.8 sharply separated the two activities, the alkaline enzyme precipitating between 20 and 25 %, and the acid enzyme between 35 and 45 % $(NH_4)_2SO_4$. Both enzymes withstood lyophilization and were almost completely inactivated upon heating at 65° for 5 min.

Washed tumor cells as well as homogenates and extracts exhibited the two pH-activity peaks mentioned above. The absolute and relative activity of the two enzymes were strikingly modified by such factors as nature or state of substrate, ionic strength of medium, and presence or absence of certain specific ions.

Fig. 1 illustrates the pH-activity curves of the two enzymes, each acting on salmon-sperm and calf-thymus DNA. The acid enzyme was much more active on thymus DNA than on the sperm preparation whereas the opposite was the case with the alkaline enzyme. It is, however, the integrity of the substrate rather than its source which determines this effect. Thus, as shown in Fig. 2, thermally degraded thymus DNA duplicated the phenomenon observed with the salmon-sperm preparation, which, based on viscosity and hyperchromic effect, was somewhat degraded. The complicated denaturation curve of the DNA is faithfully followed, in opposite directions, by the two enzymes. There was no evidence of chemical degradation of the DNA up to and including heating at 80° for 15 min, as determined by the appearance of acid-soluble material absorbing at 260 mμ.

The acid enzyme required a certain minimum ionic environment for activity, which was satisfied by a variety of ions. There was, however, some discrimination

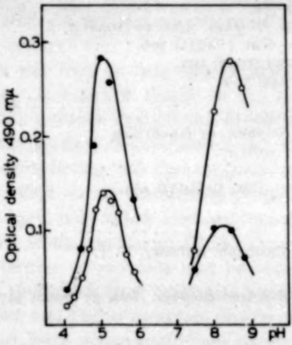

Fig. 1. pH-activity relation of acid and alkaline enzymes against salmon-sperm (○) and calf-thymus (●) DNA. Components consisted of 400 μg DNA, 300 μmoles buffer (acetate for acid, histidine for alkaline enzymes), 10 μmoles $MgSO_4$ (alkaline enzyme only), and enzyme to 1 ml: incubation 60 min at 38°. Activity was determined by the indole method of CERIOTTI as modified by KECK[3]. The acid enzyme was a particle-free saline extract; the alkaline enzyme was a washed cell suspension.

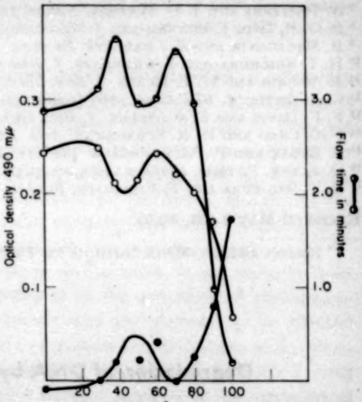

Fig. 2. Activity of ascites-tumor enzymes on heat-treated calf-thymus DNA. (○) acid enzyme; (●) alkaline enzyme; (◒) flow time of DNA determined viscometrically under standard conditions. The acid and alkaline enzymes were particle-free saline extracts. The DNA, in 0.2 M NaCl, was heated at the indicated temperatures for 15 min. Other conditions as in Fig. 1.

TABLE I

ACTIVATION AND INHIBITION OF "ACID" ASCITES DNASE*

Salt	*Ionic strength*	*Rv** × 100*
NaCl	0.001	0.0
	0.01	0.0
	0.1	4.0
	0.2	1.9
$MgCl_2$	0.0015	1.7
	0.015	1.7
	0.15	0.0
$CaCl_2$	0.0015	1.6
	0.015	1.7
	0.15	0.0

* Dialyzed, lyophilized, saline extract of tumor cells.

** Enzyme activity at pH 5.2 expressed as loss of viscosity/min of calf-thymus DNA, determined from the slope of the straight line relating flow time to sampling time (2-min intervals for 14 min) in a microviscometer at 29.5°.

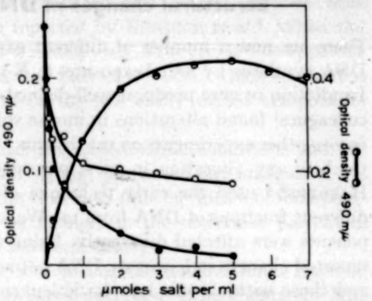

Fig. 3. Effect of divalent ions on the activity of the alkaline enzyme on salmon-sperm DNA. (○) $MnCl_2$ and (●) $CaCl_2$ inhibition curves (ordinate at left) in the presence of 1 m*M* $MgCl_2$; (◐) $MgCl_2$-activation curve. Enzyme was a dialyzed $(NH_4)_2SO_4$ fraction. Other conditions as in Fig. 1.

between monovalent and divalent ions as shown by others for DNase II[2]. Table I demonstrates that monovalent ions permitted a higher rate of activity than divalent ions, but on a concentration basis, divalent ions were more potent, both as activators and inhibitors.

The alkaline enzyme, on the other hand, was specific in its requirements for magnesium. Neither NaCl, $CaCl_2$ nor $MnSO_4$ could replace it. The latter two salts, in fact, strongly inhibited the activity of the magnesium-activated enzyme. Fig. 3 depicts the magnesium-activation curve of the alkaline enzyme as well as the inhibition curves of $CaCl_2$ and $MnSO_4$ in the presence of 1 μmole $MgCl_2$. About 1000 times more NaCl was required to achieve similar inhibition.

An enzyme of the "alkaline" type has been observed in another ascites tumor which arose spontaneously in our colony in a mouse of the Bagg strain. An enzyme with somewhat similar properties, especially as regards activation by magnesium and inhibition by calcium, has also been observed in mouse spleen and thymus, but not in normal or regenerating liver.

This work was supported in part by research grant No. CY-2903 from the National Cancer Institute, and grants from the American Cancer Society Inc. and the American Cancer Society, Southeastern Michigan Division.

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Received May 16th, 1959

Structural changes in DNA after X-radiation *in vivo*

There are now a number of different experiments which suggest that inhibition of DNA synthesis by lethal exposures to X-radiation may be due to "template" damage. Irradiation *in vitro* produces well-defined structural changes but whilst Cole and his colleagues[1] found alterations in mouse spleen and thymus nucleoprotein after 850 R *in vivo* other experiments on rat thymus DNA isolated after exposure to 1000 R failed to show any alteration in gross physical or chemical properties[2]. Backmann and Harbers[3,4] used the early technique of Bendich and his colleagues[5] to separate different fractions of DNA from rat Walker carcinoma and found that the two components were affected differently. Using a more refined fractionation Bendich[6] has reported changes in leucocyte DNA between patients with chronic myeloid leukemia and those with chronic lymphatic leukemia[7]. We have used the same procedure to analyse rat thymus DNA shortly after X-radiation.

Two experiments were performed with 32 animals/group in each case. Equal numbers of both sexes (90–120 g body weight) were used. They received 1000 R (71.6 rads/min) and were injected with ^{32}P (50 or 100 μC/0.1 ml/100 g body weight intramuscularly) immediately afterwards. The animals were killed by cervical fracture 15 min after the end of exposure, the thymus removed and dropped into liquid O_2. Unirradiated rats were similarly treated and DNA from the control and irradiated groups prepared. Deproteinisation with sodium dodecylsulphate was employed[8] but in the initial washing procedures solutions of low ionic strength were avoided[6]. In confirmation of our earlier work 1000 R produced about 50 % reduction in ^{32}P incorporation into thymus DNA (Table I); the specific activity of inorganic phosphate in the initial extract was used as reference and no difference between control and irradiated samples was found in this respect. As reported earlier the N/P ratios and ε(P)max for control and irradiated DNA were not detectably different. The DNA samples were fractionated on Ecteola columns (Type 20, Lot 1015, Brown Co., Berlin, New Hampshire, U.S.A.) using two 125-ml mixing flasks and 5-ml

TABLE I

PROPERTIES OF THYMUS DNA ISOLATED FROM NORMAL RATS AND FROM THOSE GIVEN 1000 R 15 min PREVIOUSLY

	Preparation 1		*Preparation 2*	
	Control	*1000 R*	*Control*	*1000 R*
N/P	3.40	3.35	3.58	3.62
ε (P) max	6,220	5,880	6,830	7,160
^{32}P uptake as % control	—	63.5	—	42.6
Column analysis E_{260}				
Peak 1 0.5 *M* NaCl, pH 7	1.079	1.059	0.799	0.716
2	1.600	1.669	1.594	1.456
3 2 *M* NaCl/0.1 *M* NH_3	2.159	3.352	2.359	4.161
4 2 *M* NaCl/1 *M* NH_3	4.122	4.115	5.205	4.506
5 0.5 *N* NaOH	2.276	1.380	3.045	2.190
Total E_{260}	11.236	11.575	13.002	13.029

Abbreviation: DNA, deoxyribonucleic acid.

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collections. The patterns obtained for the irradiated and control samples were qualitatively identical and similar to those reported by BENDICH *et al.*[6]. When the distribution of material within the fractions was analysed, however, it was apparent that irradiation had produced an increase in that part of the DNA which was eluted with $2\,M$ NaCl/$0.1\,M$ NH_3 at the expense of the less easily eluted components (Table I).

The low level of ^{32}P incorporation under the experimental conditions and the small amount of DNA applied to the columns (3 mg) prevented any analysis of the specific activities of the different fractions but it is clear that inhibition of incorporation produced by 1000 R is accompanied by changes in the molecular pattern of DNA which are consistent with the idea of template damage.

We are grateful to our colleagues at the Radiobiological Research Unit, Harwell, for their co-operation which enabled us to carry out these experiments, to Miss H. MINNS and Mr. J. A. MORRIS for their cheerful assistance and to the British Empire Cancer Campaign for financial support.

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Received May 2nd, 1959

Biochim. Biophys. Acta, 37 (1960) 352–353

Inhibition of the alanine-activating enzyme by rat liver "soluble" fraction RNA

As a result of the studies of a number of workers[1–7], the first 2 steps in protein synthesis have been postulated as (a) an activation of amino acids as shown in equation (1), followed by (b) a transfer of the activated amino acids to a "soluble" ribonucleic acid as shown in equation (2).

$$\text{Enzyme} + \text{Amino Acid} + \text{ATP} \rightleftharpoons \text{Enzyme–Aminoacyl–AMP} + \text{PP} \quad (1)$$

$$\text{Enzyme–Aminoacyl-AMP} + \text{RNA} \rightleftharpoons \text{Enzyme} + \text{Aminoacyl-RNA} + \text{AMP} \quad (2)$$

The possibility that RNA has some effect on the amino acid-activation step (eqn. (1)) has been mentioned in the literature. In the above formulation, RNA is not involved in this step, and it has been reported[5,6] that RNase does not inhibit this reaction

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Abbreviations: RNA, ribonucleic acid; RNase, ribonuclease; ATP, adenosine triphosphate; AMP, adenosine monophosphate; PP, inorganic pyrophosphate.

(measured as an amino acid-dependent incorporation of radioactive pyrophosphate into ATP).

However, OGATA *et al.*[8] concluded that RNA is involved in the amino acid activation step, since they observed decreased pyrophosphate incorporation after RNase treatment. Recently, NOHARA AND OGATA[9] have also found a decrease in amino acid hydroxamate formation using RNase-treated "pH 5 enzymes". A possible explanation of the difference in results has been given by VON DER DECKEN AND HULTIN[10] who found that addition of RNase directly to the reaction mixture did not inhibit the reaction, although after reprecipitation at pH 5.1 (which might involve loss of enzyme) the RNase-treated enzyme gave less incorporation.

The possibility that RNA might actually inhibit the amino acid-activation step has been suggested by the report of RENDI *et al.*[11] that yeast RNA inhibits a methionine-activating enzyme. (Yeast RNA, however, is not active in eqn. (2)[3,5]). The present note describes studies of the effects of RNase and RNA on the purified alanine-activating enzyme which has recently become available in this laboratory[12].

During studies of the alanine-activating enzyme of rat liver it was observed that the addition of RNase to a preparation of "pH 5 enzymes" which had been reprecipitated at pH 5 or pH 4.5 increased the incorporation of pyrophosphate into ATP. Typical results are shown in Table I. Therefore, when the purified alanine-activating enzyme became available, the effects of RNase and RNA upon the activation reaction were studied. The results are summarized in Table II.

TABLE I

EFFECT OF RNASE UPON ALANINE-ACTIVATING ENZYME IN ONCE-PRECIPITATED AND TWICE-PRECIPITATED "pH 5 ENZYME"

Each assay tube contained: 50 μmoles tris(hydroxymethyl)aminomethane, pH 7.5, 10 μmoles $MgCl_2$, 10 μmoles K_4ATP, 10 μmoles KF, 5 μmoles K [^{32}P]PP, 2 μmoles L-alanine where indicated, 1.0 μg RNase where indicated, 0.4 ml enzyme solution plus sufficient water to make 1.0 ml of reaction mixture. Time of incubation, 10 min at 37°. There was no pre-incubation of the enzyme with RNase. All values are averages of duplicates.

Description	*Pyrophosphate incorporation*
A Once-precipitated enzyme	
+ alanine	100*
— alanine	21
+ alanine + RNase	106
B Twice-precipitated enzyme	
+ alanine	100*
— alanine	15
+ alanine + RNase	123

* Observed values (counts/min) set equal to 100.

From the data presented in Table II it can be seen that RNase has no effect on the reaction. The reaction is inhibited by rat-liver "soluble" RNA, and this inhibition is completely reversed by RNase. Yeast RNA, however, has no effect.

The fact that the rat-liver "soluble" RNA is inhibitory, but the yeast RNA is not, and the fact that the inhibitory effect of the rat-liver RNA is overcome by RNase suggests that the inhibition is due to some special feature of the structure

TABLE II

EFFECT OF RNASE, "SOLUBLE" RNA, AND YEAST RNA UPON PURIFIED ALANINE-ACTIVATING ENZYME

The contents of each assay tube were the same as those given in Table I except that 1 µmole of 0.01 M K ethylenediaminetetraacetate*, pH 7, was added to each tube. All values are averages of duplicates.

Description	*Pyrophosphate incorporation*
Purified enzyme	
+ alanine	100**
− alanine	10
+ alanine + RNase	100
+ alanine + 0.2 mg "soluble" RNA	65
+ alanine + 0.4 mg "soluble" RNA	54
+ alanine + 0.6 mg "soluble" RNA	50
+ alanine + 0.2 mg yeast RNA	100
+ alanine + 0.6 mg "soluble" RNA + RNase***	100

* Added to the reaction mixture to prevent inhibition of the enzyme by any traces of heavy metals that might be bound to the RNA.

** Observed value (counts/min) set equal to 100.

*** The RNA + RNase mixture was preincubated 8 min at 25° before it was added to the assay mixture.

of the rat-liver RNA. Whether the inhibition is related in any way to the activity of the RNA as amino acid acceptor in eqn. (2) remains to be determined.

The authors wish to thank S. H. MERRILL for her excellent assistance.

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Received April 3rd, 1959

Bacterial tryptophan decarboxylase

In a previous communication from this laboratory[1], we reported on the tryptophan decarboxylase activity of intestinal flora obtained from a patient with idiopathic sprue. Since the occurrence of tryptophan decarboxylase in bacteria had been questioned despite the two reports in the early part of the present century[2,3], we felt it worthwhile to study this reaction in some detail.

A pure strain of the intestinal bacterium was isolated from a fresh stool sample of the patient with idiopathic sprue using conventional bacteriological techniques. The gram-positive, nonmotile organism isolated with respect to the highest tryptophan-decarboxylating activity showed the general biochemical properties characteristic of the alpha hemolytic Group D *Streptococci**. Furthermore, this organism displayed a very active tyrosine decarboxylase activity which is characteristic of *Streptococcus faecalis*.

Tryptophan-decarboxylating activity was measured as follows: 15 mg of lyophilized bacteria were incubated with 10 mg tryptophan, and 1 ml 0.1 *M* citrate buffer, pH 5.5, in a final vol. of 3 ml at 38° for 2 h. The amount of tryptamine formed was assayed by adding to the incubation mixture 5 mg of lyophilized rat-liver mitochondria as a source of monoamine oxidase, 1 ml 0.5 *M* phosphate buffer, pH 7.4, and 0.2 ml 0.2 % 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride and reincubating for 30 min.[4]. The reduced dye was extracted with 5 ml of a mixture of ethylacetate and *n*-butanol (1:1, v/v) and its absorption measured at 490 mμ. Alternatively, the xanthydrol method[1] was occasionally used as a further check. The tryptamine formed was also identified as described previously[1].

The tryptophan decarboxylase was found to be a typical bacterial amino acid decarboxylase similar to many described by GALE[5]. Thus, the optimal pH was found to be between 5.5–5.75 (Table I), the growth medium had to be acid for the appearance of the decarboxylase activity, and the acitivity of a dialyzed cell-free preparation was stimulated by the addition of pyridoxal phosphate. D-Tryptophan was not decarboxylated.

TABLE I

TRYPTOPHAN DECARBOXYLASE ACTIVITY AS A FUNCTION OF pH

pH	*µg tryptamine formed in 2 h*
5.0	83
5.3	145
5.5	174
5.75	184
6.0	158
6.5	126

Decarboxylation of tryptophan appears to occur only among the *Streptococci*, since crude preparations of commercial arginine, histidine, glutamic acid and lysine decarboxylases obtained from other species showed no activity while commercial tyrosine decarboxylase and *Streptococcus faecalis R* (A.T.C.C. 8043) were found to have

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* We are indebted to Dr. R. HUGH of the Cancer Clinic of George Washington University for characterizing this organism.

TABLE II

COMPARISON OF DECARBOXYLASE ACTIVITIES AMONG 3 DIFFERENT PREPARATIONS OF *Streptococci*

	Decarboxylase activity in μmoles/h/mg dry wt.		
	Tryptophan	*Phenylalanine*	*Tyrosine*
Commercial tyrosine decarboxylase	0.013	0.48	11.3
Streptococcus faecalis R (No. 8043)	0.020	—	12.0
Present strain	0.031	—	10.2

activity comparable to that of the present strain (Table II). It is apparent that the tryptophan decarboxylase activity is several hundred times weaker than the tyrosine activity and also much weaker than the phenylalanine activity. Because of this it was difficult to ascertain whether tryptophan decarboxylation represents a distinct enzyme. The significance of tryptophan decarboxylase from both the standpoint of the microorganism and of the human host is not apparent since the activity is so weak and the affinity for tryptophan is low ($K_m = 0.013\ M$). However, it is conceivable that under certain pathological conditions such as in chronic intestinal obstruction or in defective intestinal absorption, changes in conditions of growth of this microorganism and the amount of substrate available to it may permit significant amounts of tryptamine to be formed by this mechanism.

The skillful assistance of Mrs. F. M. DaCosta is gratefully acknowledged. We also wish to thank Dr. J. Oates and Dr. A. Sjoerdsma for making some of the clinical material available.

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Received April 6th, 1959

Biochim. Biophys. Acta, 37 (1960) 356–357

Acidic aminosugars from bacteria.
Incorporation of [1-^{14}C]-α,β-methyl-N-acetyl-D-glucosaminide into muramic acid

Lactobacillus bifidus var. *pennsylvanicus*[1] exhibits a specific growth requirement for N-acetyl-D-glucosamine, preferably in the form of its β-glycosides[2] or in the form of 4-O-β-substituted disaccharides such as N-acetyllactosamine[3] possessing N-acetyl-D-glucosamine as the terminal reducing sugar. This apparent discrepancy over different chemical structures possessing equal growth-promoting activity as well as the absolute amounts required for optimal growth (in the order of 10–20 μg/ml medium) suggested that both substances might be converted to a common intermediate engaged in a major chemical reaction of the cell. It seemed possible that N-acetyl-D-glucosamine might be necessary for the formation of the bacterial cell wall of this microorganism. This viewpoint was supported by the fact that lysozyme digests of other gram-positive microorganisms were found to possess growth-promoting activity, and by the formation of atypical forms in the absence of β-N-acetyl-D-glucosaminides, lactosamine, or human milk as is described in the following paper[4].

To explore further this hypothesis [1-^{14}C]N-acetyl-D-glucosamine was prepared[5] and converted into its anomeric mixture of methyl glycosides[2]. Under the conditions employed a consistent mixture of 85 % α- and 15 % β-methyl-N-acetyl-D-glucosaminide was obtained. Although the separation of this glycosidic mixture into the anomers had been reported previously[2], in this study no attempt was made towards their separation in the hope of finding an explanation for the previously observed "enhancing effect" of α-methyl-N-acetyl-D- glucosaminide which exhibits no growth-promoting activity *per se*.

When [1-^{14}C]α,β-methyl-N-acetyl-D-glucosaminide of specific activity 21,250 counts/min/μmole is added to the culture medium, uptake of isotope proceeds rapidly during the logarithmic phase and reaches a maximum at approx. 15 h. The uptake decreases as the total incorporation approaches 14 % of the α,β-methyl-N-acetyl-D-glucosaminide mixture which is consistent with the previous observation that only the β-anomer is active as a growth factor for *L. bifidus* var. *pennsylvanicus*. The rate of incorporation in relation to total acid production, turbidity and counts/min/mg cells is set forth in Fig. 1. Striking morphological changes in this microorganism which coincide with the availability of this growth factor are described in the following paper[4].

For the identification of the incorporated component, *L. bifidus* var. *pennsylvanicus* was grown in eight 750-ml cultures as described previously[1] in the presence of 20 μg [1-^{14}C]-α,β-methyl-N-acetyl-D-glucosaminide/ml. The cells were harvested

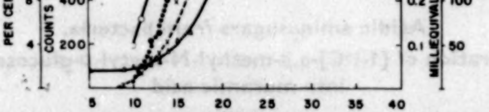

Fig. 1. *L. bifidus* var. *pennsylvanicus* was grown in series of 5-ml cultures containing 100 μg [1-^{14}C]-α,β-N-acetyl-D-glucosaminide, specific activity 21250 counts/min/μmole. All determinations were carried out in duplicate 5-ml aliquots. Curve A exhibits mequivs. acids produced. Curve B gives the turbidity as measured in a Klett colorimeter with a 560-mμ filter. Curve C represents the per cent uptake of isotope as measured by the total number of counts in the isolated cells. Curve D represents the counts/min/mg (dry weight) cells. Radioactivity was counted in a windowless gas-flow counter at infinite thinness.

after 25 h, washed once with distilled water, suspended in 300 ml 0.005 M phosphate buffer, pH 7.0, and treated with 30 mg lysozyme (Armour) in the presence of 500 mg ethylenediaminetetraacetic acid and 1 M sucrose for 32 h at 37°. After this treatment electron-microscopic examination revealed approx. 30–40% protoplast formation, the remaining organisms showing damaged cell walls. The supernatant fluid of the lysozyme digest obtained by centrifugation was fractionated by ion-exchange chromatography on IR120 (H^+) and Dowex I in the formate and (OH^-)-forms.

The damaged cells and protoplasts were osmotically shocked with distilled water and centrifuged. The cellular débris thus obtained, consisting chiefly of membrane and cell-wall material, was hydrolyzed with 2.5 N HCl at 100° for 4 h under reflux. After evaporation of the filtered hydrolyzate to dryness, the residue was dissolved in water, adjusted to pH 6, adsorbed on charcoal and successively eluted with water and increasing concentrations of aqueous ethanol. The 10% ethanolic fractions were combined and rechromatographed on a charcoal–celite (1:1) column and eluted with water–acetic acid as the gradient (100 ml 15% (v/v) acetic acid into 200 ml water). The acetic acid eluates containing most of the total radioactivity were combined, evaporated to dryness and further purified by means of paper electrophoresis at pH 6.5 (in pyridine–acetic acid–water, 100:10:890) and at pH 1.9 (in formic acid–acetic acid–water, 50:150:800).

As can be seen from Fig. 2 which exhibits the distribution of radioactivity with reference to mobility at 25 V/cm, the newly formed component behaves like a Zwitterion, being immobile at pH 6.5 and traveling cathodically at pH 1.9. Elution of the electroneutral zone from the electropherogram at pH 6.5 and electrophoresis again at pH 1.9 yielded after elution a substance which exhibited positive reducing, ninhydrin and ELSON-MORGAN tests. When the last was performed according to the

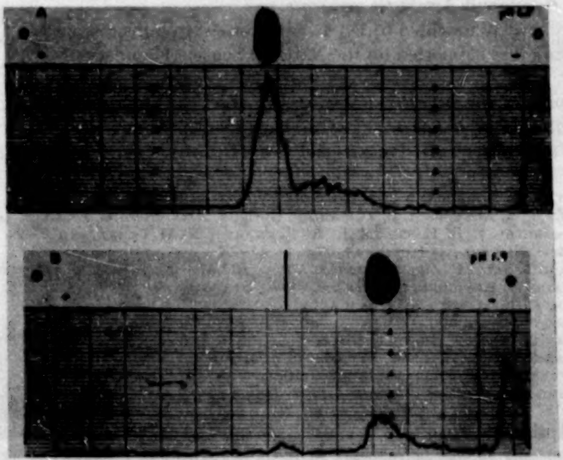

Fig. 2. Electrophoresis of partially purified muramic acid obtained from membranes and cell walls of *L. bifidus* var. *pennsylvanicus* (a) at pH 6.5; (b) at pH 1.9 (Whatman No. 1. paper; 25 V/cm). The radioactive zones were detected with a paper-strip scanner (Nuclear-Chicago) and coincide with the reducing substance at both pH values.

modification of Boas[6] a spectrum typical for 3-O-substituted hexosamines (λ max. 512 mμ) was obtained. Paper chromatography of this substance in pyridine–ethyl acetate–acetic acid–water (5:5:1:3) revealed a spot with mobility identical to synthetically prepared 3-O-D-lactyl-D-glucosamine[10] ($R_{\text{glucosamine}} = 1.1$) exhibiting positive tests with ninhydrin, aniline oxalate and the Elson-Morgan reagent. On the basis of a quantitative Elson-Morgan test (Boas modification[6]) with synthetic 3-O-lactyl-D-glucosamine as a standard, the specific activity of the isolated muramic acid was found to be 17,100, 18,334 and 25,522 counts/min/μmole as compared with 21,250 counts/min/μmole of the starting material.

From the lysozyme digest a substance was isolated exhibiting properties similar to the above-described acid, but in very small amounts. The relative insensitivity of *L. bifidus* var. *pennsylvanicus* towards lysozyme[7] may account for the low yield in the latter experiment.

Our findings demonstrate that N-acetyl-D-glucosamine is a direct precursor in the biosynthesis of muramic acid, or more precisely of 3-O-D-lactyl-D-glucosamine previously isolated[8] and identified[9] from hydrolyzates in 6 *N* acid of spore peptides and bacterial cell-wall preparations. However, no conclusion may be derived from this observation as to the true chemical structure of the naturally occurring form of muramic acid, particularly since synthetically prepared 3-O-D- or 3-O-L-lactyl-N-acetyl-D-glucosamine[10] did not exhibit any growth-promoting activity for this microorganism. The biosynthesis of muramic acid, which probably proceeds at the level of phosphorylated intermediates, is at present under investigation using a cell-free enzyme system derived from the same microorganism. In this connection it is noteworthy that Strominger[11] recently indicated a pyruvic acid transfer from phosphoenol pyruvate to uridine diphosphate N-acetyl-D-glucosamine with extracts of *S. aureus*, *E. coli* and *Aerobacter aerogenes*.

We are greatly indebted to Dr. T. Sall for electron microscopy.

The work was supported by the National Science Foundation.

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Received April 10th, 1959

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Morphological changes of *Lactobacillus bifidus* var. *pennsylvanicus* produced by a cell-wall precursor

Atypical forms of microorganisms have been produced by selective interference with the biosynthesis of specific cell-wall constituents. Bizarre forms have been obtained by growth in the presence of penicillin[1] or high concentrations of glycine[2]. In the former case, growth in the presence of penicillin resulted in inhibition of cell-wall synthesis and in the accumulation of intermediates such as peptides of uridine diphosphate muramic acid[3]. Omission from the medium of specific cell-wall precursors, such as the amino acids, α, ε-diaminopimelic acid[4] or lysine[5] during the growth of organisms requiring these substances also results in the formation of a typical forms and lysis.

We wish to describe similiar morphological changes specifically produced by an amino sugar. The preceding paper[6] has demonstrated α- β-methyl-N-acetyl-D-glucosaminide to be precursor of muramic acid or 3-O-D-lactyl-D-glucosamine, which has been isolated from bacterial cell walls[7].

Lactobacillus bifidus var. *pennsylvanicus*, a mutant requiring human milk, or more specifically N-acetyl-D-glucosamine-containing saccharides, was first isolated from feces of breast-fed infants[8]. This microaerophilic organism is cultured on a modified TEPLY AND ELVEHJEM medium[8] containing 2% skimmed human milk. Under these conditions this mutant appears as a slender bifid rod. The organism exhibits a similar appearance when grown in the presence of the following synthetic growth factors in amounts previously described as optimal[8]: α, β-methyl-N-acetyl-D-glucosaminide, 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine, and N-acetyl-D-glucosamine.

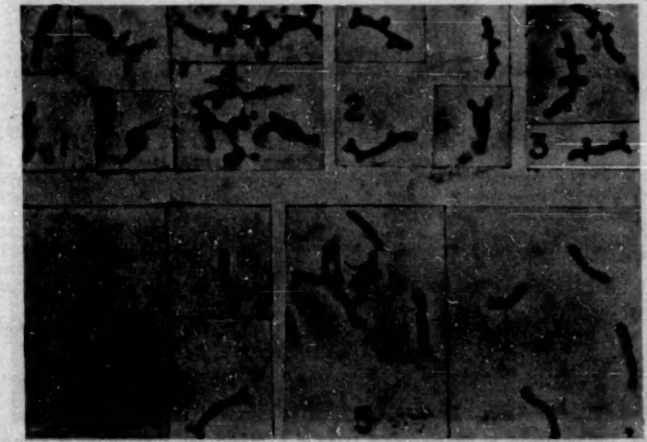

Fig. 1–5. The succession of morphological types of *Lactobacillus bifidus* var. *pennsylvanicus* grown for 40 h in 5 ml medium in the presence of serial amounts of α, β-methyl-N-acetyl-D-glucosaminide at 37° in 90% N_2, 10% CO_2. The pictures were taken of wet mounts of the living cells in the Anoptral microscope of Reichert, Vienna. Final magnification 1730 ×.
Fig. 1. no growth factor; Fig. 2, 50 µg; Fig. 3, 100 µg; Fig. 4. 200 µg; Fig. 5. 400 µg.

In these experiments, *L. bifidus* var. *pennsylvanicus* was grown in duplicate series of 5-ml cultures with varying amounts (10–1000 µg) of α, β-methyl-N-acetyl-D-glucosaminide as growth factor, under an atmosphere of 90 % N_2, 10 % CO_2 at 37° for 40 h. Figs. 1–5 show the succession of morphological types which developed, varying from the bulbous forms when grown with little or no amino sugar through knobbed forms, to simple rods completely lacking the bifid structure when cultured in the presence of high concentrations of this growth factor. Similar changes were obtained with serial amounts of the other substances mentioned above, including skimmed human milk.

The preceding paper[6] described the incorporation of [1-^{14}C]-α,β-methyl-N-acetyl-D-glucosaminide into muramic acid. Fig. 1 of that paper correlated the rate of incorporation, development of turbidity and acid in the cultures. The morphological sequence in one such experiment is shown in Figs. 6–12. During the early growth phase (Figs. 6, 7) when sufficient amounts of the α, β-methyl-N-acetyl-D-glucosaminide were available, the organisms appeared as rods. In the later phase (Figs. 10, 11, 12)

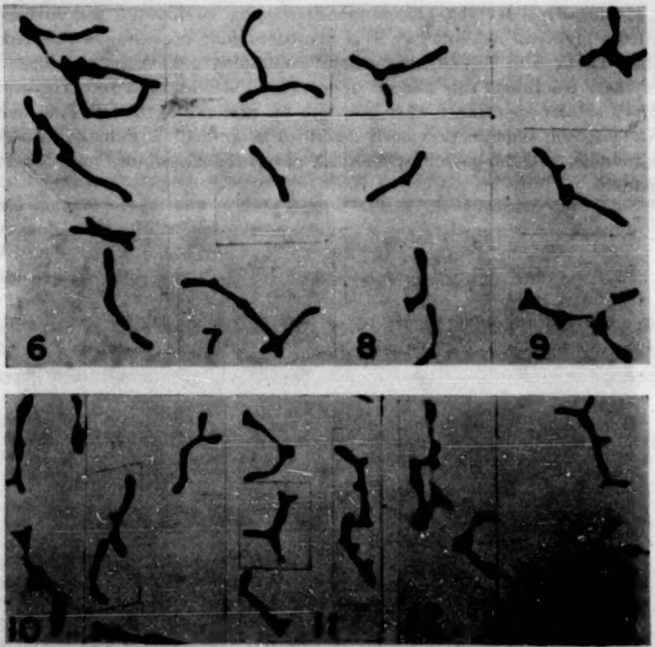

Fig. 6–12. The succession of morphological types of *L. bifidus* var. *pennsylvanicus* grown with 100 µg α, β-methyl-N-acetyl-D-glucosaminide in 5 ml medium at 37° in 90 % N_2, 10 % CO_2. At intervals, smears of the culture were made, air dried, heat fixed and stained with 0.25 % aqueous crystal violet for 2 min. Photographs with KAMV camera attached to Reichert Neozet microscope with transmitted light. Final magnification 1730 X. Fig. 6–12 corresponding to 14, 15, 16.5, 20, 24, 30 and 47-h growth.

after maximum incorporation, when the available glucosaminide had been extensively utilized, the organisms began to branch and finally to assume the form characteristic for 100 μg of growth factor as shown in Fig. 3.

The bulged forms of *L. bifidus* var. *pennsylvanicus* obtained when grown for 20 h in the presence of suboptimal amounts of growth factor disappeared upon addition of more α,β-methyl-N-acetyl-D-glucosaminide and rods were observed.

The formation of atypical forms in *L. bifidus* was first observed by TISSIER[9] and more recently by ORLA-JENSEN[10], PETUELY[11] and SUNDMAN AND BJÖRKSTEN[12]. The latter workers described globular forms of bifid lactobacilli on a medium permitting abundant growth The development of these bladder-like forms could be prevented by the addition of a tryptic digest of cow's milk.

Thus, we have been able to demonstrate the formation of bizarre forms in *L. bifidus* var. *pennsylvanicus*, resulting from the deficiency of an N-acetyl amino sugar which is an essential growth requirement and a precursor for cell-wall synthesis. Furthermore, even the bifid character of the organism may be a consequence of an inadequate supply of this same factor, since we can eliminate branching by elevating its concentration.

We are indebted to Dr. R. F. NORRIS for making available the Woodward Fellowship to one of us (M.C.G.).

This work has been aided by a grant from the U.S. Atomic Energy Commission, AEC Contract No. AT(30-1)-1342.

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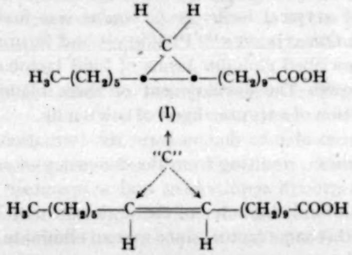
Received April 10th, 1959

Lactobacillic acid biosynthesis

In previous communications[1, 2] we have presented indirect evidence in support of our concept that the biosynthesis of lactobacillic acid (I), by lactic acid organisms, may involve the addition of a one-carbon fragment across the double bond of *cis*-vaccenic acid (II).



(I)

"C"

(II)

We now wish to record direct experimental evidence in support of this rather novel biochemical reaction.

Lactobacillus arabinosus was grown in presence of [1-^{14}C]*cis*-vaccenic acid ($1.35 \cdot 10^7$ disintegrations/min/µmole) in a medium low in biotin[1] and the fatty acids were isolated from the washed bacterial cells. The fatty acid mixture was hydroxylated, separated into its components[3] and the radioactivity of individual fatty acids was determined. Of the added [1-^{14}C]*cis*-vaccenic acid, 18.7 % was incorporated into the cells; 99 % of the radioactivity was located in the fatty acids. This radioactivity was distributed between the "dihydroxy" and lactobacillic acid fractions; the other fatty acids were inactive (Table I). It is worthy of note that the specific activities of the "dihydroxy" and lactobacillic acid fractions were 82 and 92 % respectively that of the [1-^{14}C]*cis*-vaccenic acid initially added to the medium. These results, which agree with previous observations[4], demonstrate incorporation of *cis*-vaccenic acid into the bacterial cells and its direct conversion into lactobacillic acid. The high specific activity of the cellular *cis*-vaccenic acid (dihydroxy fraction) suggests that the bacteria failed

TABLE I

INCORPORATION OF LABELLED PRECURSORS INTO *L. arabinosus* FATTY ACIDS*

	Weight (mg) and radioactivity of fatty acids						
Precursor	*"Dihydroxy"*	*C10*	*C12*	*C14*	*C16*	*C19*	*Total fatty acids*
[1-^{14}C]*cis*-vaccenic acid	9.35 1.10×10^7**	0.10 —	0.14 —	0.48 —	25.3 —	20.95 1.24×10^7**	61.0
Sodium [^{14}C]formate	2.7 165***	0.03 —	0.04 —	0.15 —	7.4 45***	5.76 1162***	17.1 1550***

* See ref.[3] for interpretation of the various chromatographic fractions.
** disintegrations/min/µmole.
*** disintegrations/min in total sample.

to produce significant amounts of *cis*-vaccenic acid under our experimental conditions.

Formate incorporation into *L. arabinosus* fatty acids was demonstrated in a second series of experiments in which sodium [^{14}C]formate ($3.8 \cdot 10^6$ disintegrations/min) was incubated with a cell suspension (5 g wet wt.) in 0.03 *M* phosphate, pH 6.8 (50 ml) containing 1 % glucose. The total fatty acids isolated from these cells exhibited a radioactivity of 1550 disintegrations/min. On chromatography, the major proportion of this radioactivity accumulated in the lactobacillic acid fraction (Table I).

The radioactivity present in the dihydroxy fraction was located in a sharp band preceding the major dihydroxyoctadecanoic acid peak suggesting that an intermediate in the conversion of *cis*-vaccenic into lactobacillic acid may be involved.

The predominant incorporation of formate carbon into lactobacillic acid is in excellent agreement with our hypothesis[1] pertaining to lactobacillic acid biosynthesis. Experiments to locate the radioactivity of the biosynthetic lactobacillic acid in the methylene-bridge carbon atom are in progress. A procedure for this purpose has been described recently[5].

This work was supported by a grant from the U.S. Public Health Service.

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Received May 19th, 1959

Biochim. Biophys. Acta, 37 (1960) 364–365

The synthesis of D-arabinose-5-phosphate

A chemical source of D-arabinose-5-phosphate was needed for studies of the enzymic dissimilation of this compound by *Propionibacterium pentosaceum*. Early work concerned with the enzymic formation of A-5-P was hampered by the difficulty in obtaining or preparing the compound. The synthesis reported by LEVENE AND CHRISTMAN[1] involved the formation of 7 intermediary compounds between the starting material, D-arabinose, and the product. The synthesis reported here is a simple 2-step procedure for the synthesis of A-5-P from glucosamine.

Glucosamine-6-phosphate was prepared by phosphorylation of glucosamine by yeast hexokinase as described by BROWN[2]. The reaction mixture for the synthesis of A-5-P contained 400 µmoles glucosamine-6-phosphate, 1000 µmoles triketohydrindene hydrate, and 1500 µmoles citrate buffer, pH 4.7, in a final vol. of 60 ml. The yield of A-5-P remained constant between pH 4.2 and 5.2, but decreased markedly on either side of this pH range. The reaction mixture was placed in a boiling-water bath. Pentose formation was followed by means of the orcinol test[3]. As shown in Fig. 1 a maximum yield of pentose was obtained after a 40-min period at 100°.

Abbreviation: A-5-P, D-arabinose-5-phosphate.

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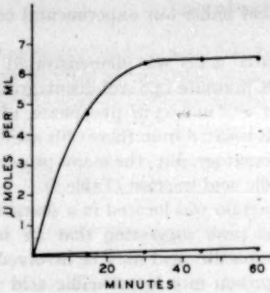

Fig. 1. The system contained 400 µmoles glucosamine-6-phosphate, 1500 µmoles citrate buffer, pH 4.7, and 1000 µmoles triketohydrindene hydrate in a total vol. of 60 ml. The mixture was placed in a boiling-water bath and the amount of pentose formed was determined at times indicated. Free pentose was determined after adsorption of phosphorylated pentose on Dowex-1 formate. ● total pentose; ○ free pentose.

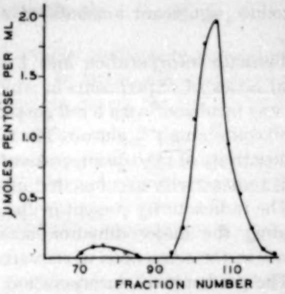

Fig. 2. The reaction mixture contained 400 µmoles glucosamine-6-phosphate, 1500 µmoles citrate buffer, and 1000 µmoles triketohydrindene hydrate in a total vol. of 60 ml. After heating at 100° for 40 min, the reaction mixture was cooled and adsorbed directly on a Dowex-1 formate column. Elution was achieved with 0.2 *N* formic acid containing 0.02 *M* ammonium formate. ○ Fraction 1; ● Fraction 2.

At the end of the heating period, the dark-blue mixture was cooled and adsorbed on a Dowex-1 formate column (2 cm × 20 cm). (It should be noted that considerably larger amounts can be handled if one uses a larger Dowex-1 formate column or if the A-5-P is precipitated at this point as its alcohol insoluble barium salt and then redissolved and adsorbed on a Dowex-1 formate column.) The column was eluted with 0.2 *N* formic acid containing 0.02 *M* ammonium formate. Fig. 2 illustrates the elution pattern of the pentoses. It can be seen that the phosphorylated material giving a positive orcinol test was eluted in 2 fractions. Fraction 1 comprised less than 1 % of the total pentose and is believed to contain both ribose and fructose phosphates. The material in Fraction 2 (334 µmoles pentose phosphate) was pooled and neutralized to pH 7.5 with KOH. $BaCl_2$ (1000 µmoles) and ethanol (to a final concn. of 80 %) were added. After overnight at 2°, the barium salt was collected by centrifugation and dissolved in water. The total amount of pentose phosphate at this point was 197 µmoles.

In order to identify the pentose phosphate in Fraction 2, 2000 µmoles glucosamine-6-phosphate were treated as outlined above. After chromatography on a Dowex-1 formate column, Fraction 2 (1670 µmoles) was pooled and precipitated as the alcohol-insoluble barium salt. After overnight at 2°, the precipitated barium salt was collected by centrifugation, dissolved in water, freed of barium by the addition of K_2SO_4, and treated with acid phosphatase in acetate buffer, pH 5.0, for 4 h. At this time the reaction mixture was made 0.005 *M* with $Na_2B_4O_7$, adjusted to pH 8.0, and adsorbed directly on a Dowex-1 borate column (1 cm × 15 cm). The column was eluted with 0.02 *M* $Na_2B_4O_7$, and 20-ml fractions were collected. The single pentose fraction was pooled, passed through a Dowex-50 (H^+) column to convert the borate to boric acid, and lyophilized to dryness. The boric acid was removed as the methyl borate[4]. The diphenylhydrazone of the pentose was prepared from the residual syrup according to the procedure of BROWNE[5]. The m.p. of the diphenylhydrazone was

203°. Authentic D-arabinose diphenylhydrazone melted at 202°, and the mixed m.p. was 202°.

Since the arabinose phosphate was synthesized from glucosamine-6-phosphate the phosphate on the arabinose would be expected to be limited to the 5 position. This conclusion is supported by the observation that only 16 % of the phosphate of the synthesized arabinose phosphate was hydrolyzed in 1 h in 1 N H_2SO_4 at 100°.

This work was supported in part by a grant, A907, from the Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service, National Institutes of Health.

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Received April 6th, 1959

Biochim. Biophys. Acta, 37 (1960) 365–367

A colorimetric method of estimation of shikimic acid*

Procedures for the estimation and detection of shikimic acid, involving oxidation by periodate, have been reported[1-3]. During periodate oxidation of shikimic acid, formation of a conjugated aldehyde occurs[4]. Since thiobarbituric acid selectively couples with conjugated aldehydes to produce colored products[5], it appeared feasible to devise a method of estimation of shikimic acid based on periodate oxidation and subsequent reaction with thiobarbituric acid. The reagents employed have been previously described[6], and the behavior of various aldehydes upon reaction with thiobarbituric acid has been reviewed[5].

Mix 0.5 ml of a solution of 0.025 M HIO_4 in 0.125 N H_2SO_4 with 3.5 ml of an aqueous solution containing 2.5–60.0 μg of shikimic acid** in a test tube. Heat the tube in a water bath at 56° for 60 min. Remove the tube, add 1.0 ml of 2 % reagent-grade $NaAsO_2$ in 0.5 N HCl, and mix well. After 2 min, transfer an aliquot of 1.0 ml, or less, of the resulting solution to a test tube containing 2.0 ml 0.6 % thiobarbituric acid (pH 2.0). Bring the volume to 3.0 ml with distilled water and mix the contents of the tube. Prepare a blank by adding 1.0 ml of distilled water to 2.0 ml of the thiobarbituric acid reagent. Heat the tubes for 60–65 sec in a boiling-water bath and immediately immerse them in cold water. After 1–5 min, add 0.3 ml 1.0 N NaOH, mix well, and within 3 min, immerse the tubes for 60–65 sec in a boiling-water bath. Cool the tubes in cold water for 1 min and measure the intensity of the blue solution at the absorption maximum of 660 mμ (Fig. 1) with a Beckman DU spectrophotometer.

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* This paper is taken from a dissertation to be submitted by L. D. SASLAW to the Graduate School of Georgetown University in partial fulfillment of the requirements for the Ph. D. degree.
** Obtained from Mann Research Laboratories, Inc., New York, N.Y.

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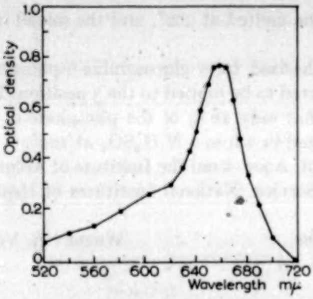

Fig. 1. Visible spectrum obtained from periodate reaction mixture and thiobarbituric acid. The concentration of shikimic acid was 10.0 μg/ml.

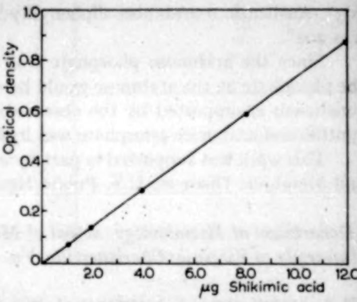

Fig. 2. Relationship between concentration of shikimic acid and absorbancy. The reactions were performed as described in the text.

The color fades rapidly on prolonged exposure to light and the tubes should be stored in the dark if absorbancy measurements are not to be made immediately. The decrease in the absorbancy, after 15 min and 30 min, in the dark, was 2 % and 4 % respectively. The absorbancy was proportional to concentration up to at least 12.0 μg/ml (Fig. 2). The reproducibility of the method was found to be ± 5 %. An equivalent amount of D-quinic acid[6] yields less than 3 % of the absorbancy observed with shikimic acid.

The arsenite-reduced solutions may be stored at 4° for 3 days. HIO_4 oxidation, and destruction of excess HIO_4, may be carried out with a 5-fold decrease in the volume of each solution used so that the 1.0 ml obtained may be heated directly with 2.0 ml thiobarbituric acid. In practice, the 5.0 ml obtained after arsenite addition may be used for replicate and ancillary determinations.

The method is approximately 20 times as sensitive as another colorimetric procedure for shikimic acid[3].

Periodate oxidation, followed by colorimetric assay with thiobarbituric acid, has been employed by WEISSBACH AND HURWITZ for estimation of 2-keto-3-deoxyphosphoheptonic acid[7]. This compound has been proposed as an intermediate in the formation of shikimic acid[8]. Since the same reagents are employed for estimation of shikimic acid and 2-keto-3-deoxyphosphoheptonic acid, the present method is of further advantage in studying the conversion of the latter to shikimic acid.

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Received May 1st, 1959

Preliminary Notes

Effects of chloramphenicol on RNA synthesis in *Escherichia coli* irradiated with ultraviolet light

In a previous communication[1] it was reported that in the cells of *E. coli* which have received ultraviolet irradiation, the synthesis of a stable RNA is observed during the first 30-min incubation, followed by the synthesis of unstable RNA.

Other authors[2–5] have suggested that the mutation induction by *E. coli* is intimately dependent on the post-irradiation synthesis of protein. It is thus of interest to study the biosynthetic relationship between RNA and protein with special attention to the heterogeneity of RNA. The dependence of the DNA synthesis upon the post-irradiation synthesis of protein has already been reported by HAROLD AND ZIPORIN[6].

The cells of *E. coli* B(H) were harvested during their logarithmic phase by centrifugation and resuspended in *tris*(hydroxymethyl)aminomethane medium and irradiated as described previously[1]. In all experiments the dose was so chosen as to give survivals of about 0.1 %. After irradiation the cells were resuspended in fresh *tris*(hydroxymethyl)aminomethane-glucose medium and incubated at 37°. To this culture chloramphenicol (40 μg/ml) was added at the times indicated and the amount of RNA was measured by the orcinol reaction upon the Schmidt-Thannhauser fractions. The post-irradiation incubation was carried out under reduced light in order to prevent photoreactivation. A typical result is shown in Fig. 1.

It will be seen that (a) when chloramphenicol is added at zero time, subsequent

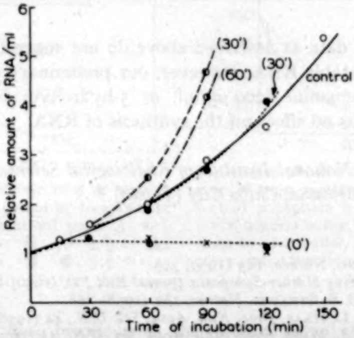

Fig. 1. Effects of chloramphenicol on the RNA synthesis by *Escherichia coli* B(H) irradiated with ultraviolet light. The figures in the parentheses show the time at which chloramphenicol is added to the culture. 0 min (---⊕---), 30 min (——◐——, ---●---), 60 min (—·—◑—·—), and control (non-irradiated cells wihout chloramphenicol) (—○—). The data when chloramphenicol is washed out at 30 min are also shown (×).

Abbreviation: RNA, ribonucleic acid.

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synthesis of RNA is inhibited nearly completely for about 120 min, and (b) when the drug is added after 30 min or later no such an inhibition is observed but rather a considerable stimulation was observed in several experiments. These results suggest that the synthesis of both kinds of RNA are equally dependent on the post-irradiation protein synthesis. Even in the experiments where the contact of the cells with chloramphenicol was limited to the first 30 min, subsequent synthesis of RNA was also inhibited for 60 min or longer. Thus the effective protein synthesis probably finish during the first 30-min incubation.

The stability of the RNA synthesized after 30 min in the presence of chloramphenicol was also studied. The irradiated cells were incubated for 30 min and then chloramphenicol and ^{32}P were added to the culture. The incubation was continued for another 30 min and after this unlabeled phosphate was added to reduce the isotope concentration to one hundredth and the culture was incubated further. It was found (Table I) that after the addition of unlabeled phosphate, ^{32}P in the RNA fraction was retained much more than in the control where no chloramphenicol was added. Thus the RNA synthesized in the presence of chloramphenicol is not identical to the labile RNA reported previously[1].

TABLE I

THE STABILITY OF THE RNA SYNTHESIZED IN THE PRESENCE OF CHLORAMPHENICOL BY ULTRAVIOLET-IRRADIATED *Escherichia coli* B(H)

Times after the addition of ^{32}P min	*no chloramphenicol added (counts/min/ml)*	*chloramphenicol added (counts/min/ml)*
0	2950	2400
30	1440	3110
60	1050	3310
120	1050	2520

The experimental data as described above do not suggest any relation between the stable and the unstable RNA. However, our preliminary data have shown that the addition of 8-azaguanine (200 μg/ml) or 5-hydroxyuridine (50 μg/ml) to the culture at zero time has no effect on the synthesis of RNA.

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Received October 29th, 1959

Biochim. Biophys. Acta, 37 (1960) 369–370

Some relations between enzyme activity, chemical reactivity and urea-induced disorientations in ribonuclease

Ribonuclease reacts with bromoacetic acid to form a stable, inactive derivative carrying a single carboxymethyl group; this group is located on a particular histidine residue, which appears to form part of the active centre of this enzyme[1,2]. A similar inactivation involving a histidine residue occurs with iodoacetic acid[3]. The active-centre histidine is activated towards the reagent[2]: it is clearly of enzymological interest to examine the structural requirements for this reactivity.

To investigate the non-covalent features of the structure, phenomena involved in the unfolding of RNase in urea solution, as documented by ANFINSEN and co-workers, are of much value[4–6]. In this context, we have observed that, while all the enzymic activity can be recovered after exposure to urea at 37° at pH 5–7, an irreversible inactivation sets in at about pH 9, and is considerable at pH 10.6 (Fig. 1). Maximum possible reversal, prior to contact with RNA in the assay[7], was obtained by dilution (finally 0.05 *M* urea) and incubation with phosphate (0.2 *M*, 37°, 5 min) in all the present experiments. No appreciable inactivation occurred (up to pH 10.6) in parallel controls without urea.

RNase is inactivated, relatively rapidly, by bromoacetate in 8 *M* urea at pH 5.3 (Fig. 2). After 90 min at pH 5.5, the isolated protein showed no loss of tyrosine in an acid hydrolysate, but there was a loss of 1.2 ± 0.1 of the 4 histidines. However, there was no significant loss of histidine (or tyrosine) in RNase reacted similarly with bromoacetate in urea at pH 5.5, when this reaction was preceded by inactivation in 8 *M* urea alone at pH about 10.5 (37°, 13 h). Hence, the bromoacetate reaction in urea appears to occur at the same site as in water, but can be abolished by sufficient disorientation.

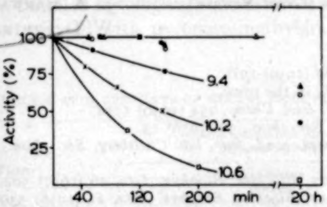

Fig. 1. Inactivation of RNase in 8 *M* urea at 37°. pH ranges (dilute acetate or bicarbonate buffers and/or manual titration) were: △—△, 5.4 ± 0.1; ■, 5.8 ± 0.1; ◓, 7.2 ± 0.1; ▲, 8.2 ± 0.2; ○, 8,7 ± 0.1; ●—●, 9.4 ± 0.2; ×—×, 10.2 ± 0.3; □—□, 10.6 ± 0.1.

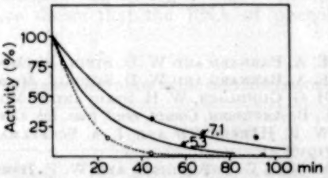

Fig. 2. Inactivation of RNase (0.16 mg/ml) by 0.31 *M* bromoacetate in 8 *M* urea, 37°: ●, in 0.12 *M* phosphate buffer, pH 7.1; ○---○, in 0.1 *M* acetate buffer, pH 5.3. (After pre-incubations in urea/buffer, 50 min.) Solid lines show the parallel reactions[3] *without* urea, at pH 5.3 and 7.1.

Combination of these results with the data of other authors permits several stages to be distinguished in the effects of urea on RNase:

1. Minimal changes in physical properties in urea occur in the presence of phosphate or similar anions, and presumably of RNA[6]. However, some unfolding is detected[6]; this does not affect the bromoacetate reactivity of the active-centre

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Abbreviations: RNA, ribonucleic acid; RNAse, ribonuclease.

histidine (Fig. 2). The hydrolysis of RNA shows some inhibition when measured titrimetrically[8], but none by assay of dialysable nucleotide[4]. Assays of acid-soluble nucleotide must be disturbed by the change of precipitability of the products in urea[4,6,8], but the results clearly indicate considerable enzyme activity in 8 *M* urea. Assays based on changes in physical properties of RNA are complicated by changes in those due to the urea itself[9].

2. In the absence of those ions, 8 *M* urea (at pH 5 to 7) produces marked unfolding[5,6]. This is fully reversed on removal of urea, and the activity is then unimpaired (Fig. 1). In urea (3.2 *M*[8] and 8 *M*[10]) the activity towards nucleoside cyclic phosphates is considerably reduced. Re-folding by these, at the concentrations employed, must be less than with RNA. However, the active-centre histidine reacts normally (apparently even faster) with bromoacetate. (Spectral changes confirm, *inter alia*, that no refolding occurred in urea in the media used here, nor with bromoacetate.) Two possible alternative explanations are that a part of the catalytic site is not required for the activation of this histidine, *or* that a separate specificity site exists and is modified or separated here, *i.e.* substrate binding is selectively decreased.

3. As the pH is raised in 8 *M* urea at 37°, a slow, *irreversible* enzymic inactivation sets in (Fig. 1). In this case, reversal cannot be achieved even with RNA (or phosphate) in water. The reactivity of the histidine is simultaneously lost. Hence, this reactivity must derive from structural features other than the amino acid sequence alone.

Analysis of the data suggests a group of apparent p*K* of roughly 10.5 in urea may be concerned in the irreversible process, but the evidence does not justify interpretation of the activity changes in terms of a single process. Rather, taken with titration data in urea[11], it supports the view (*cf.* SAGE AND SINGER[12]) that ionisation of all 6 tyrosines in RNase is followed by a slow, secondary, irreversible inactivation.

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Received September 19th, 1959

RNA fractions in cell nuclei isolated by phenol and by sucrose-glycerophosphate

In our previous reports[1–4] the association of certain protein and nucleoprotein fractions of cell nuclei with definite nuclear structures was described, the acid protein fraction representing the material of the nucleoli and residual chromosomes.

The distribution of nuclear material among these fractions shows that in nuclei with a more highly developed nucleolar apparatus there is a higher percentage of the acid protein fraction and correspondingly a higher acid protein/DNA ratio (Table I). The nuclei were isolated from different tissues by a two-step modification of the previously described sucrose-glycerophosphate method[3].

We also isolated apparently pure nuclei by a procedure based on a previous work of one of us[5] in which it was shown that the result of the action of phenol on nucleoproteins at different pH's depends mostly on the type of protein in the nucleoprotein complex. The DNA-protein formed in an intermediate layer between the phenol and water phases, after treatment of the tissue homogenate with phenol (pH 6)–0.14 M NaCl appears under the microscope as nuclei. For the purification of the nuclei, the intermediate layer was shaken with fresh phenol–NaCl and centrifuged at low speed for 20–30 sec. Nuclei free from cytoplasmic contamination remained in the aqueous phase. After a few similar treatments of the remaining intermediate layer, pure nuclei were concentrated and washed 3–4 times with the phenol–NaCl mixture.

Such phenolic nuclei appear to represent the material of nucleolar–chromatin complex of nuclei, the DNA content being higher than in sucrose-glycerophosphate nuclei (Table I). The RNA content and RNA/DNA ratio shows a good correlation with the development of the nucleolar apparatus.

Detailed cytological studies on nuclei from Ehrlich ascites carcinoma and rat liver, stained with pyronine–methylgreen have shown that the RNA of phenolic

TABLE I

NUCLEIC ACID CONTENTS OF NUCLEI ISOLATED BY DIFFERENT METHODS FROM VARIOUS TISSUES

Tissue	*Nuclei isolated with sucrose-glycerophosphate*			*Phenolic nuclei*		
	DNA %	*acid protein fraction %*	$\frac{\text{acid protein}}{\text{DNA}} \times 10$	*DNA %*	*RNA %*	$\frac{\text{RNA}}{\text{DNA}} \times 100$
Sturgeon spermatozoa	42.2	4.1*	0.97	—	—	—
Rat kidney	—	—	—	36.0	0.32	0.89
Calf thymus	—	—	—	35.5	0.72	2.03
Rat spleen	25.2	5.5	2.2	35.0	0.79	2.26
Rat liver	22.2	7.5	3.4	28.1	2.33	8.30
Hen liver	19.4	12.1	6.2	—	—	—
Rat brain	19.3	13.1	6.8	28.3	3.30	11.70
Hen embryonic liver	17.8	16.2	9.1	—	—	—
Mouse Ehrlich ascites carcinoma	13.6	22.5	16.5	28.3	4.22	14.90

* In contrast to acid proteins of other tissues, containing 10–20 % of RNA, the sperm-head acid protein is very poor in RNA (2 %).

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

nuclei is the RNA of nucleoli and residual chromosomes[1-4] or RNA$_2$[6-8]. Nuclear sap RNA (RNA$_1$) was removed from nuclei by the phenolic treatment. Thus, determination of the RNA/DNA ratio in phenolic nuclei may be a convenient measure of the development of the nucleolar apparatus.

Phenolic nuclear RNA (probably the RNA$_2$[6-8]) was further fractionated with the aid of phenol (pH 6)–distilled water or phenol (pH 8.3-8.4)–0.14 M NaCl. Approximately 2/3 of the RNA was liberated in the aqueous phase (RNA-fraction "E") and 1/3 remained in the nuclear residue (RNA-fraction "N").

Table II represents the incorporation of ^{32}P in different RNA fractions of ascites cells of Ehrlich carcinoma *in vitro*. It is evident that the most active fraction is RNA-N, followed in activity by RNA-E. It is probable that the high incorporation of ^{32}P in RNA non-extractable by Kirby's procedure[9], reported by SIBATANI *et al.*[10], is due to the presence of "phenolic nuclei".

TABLE II

The cells were incubated *in vitro* for 1 h at 37° with ^{32}P added to ascites fluid (10 μC/ml). The specific activity of RNA nucleotides was measured after alkaline hydrolysis and adsorption/elution on "Norit"[11]. In Expt. No. 2, RNA-E was isolated with phenol (pH 8.3–8.4)–0.14 M NaCl; in Expt. No. 3 with phenol(pH 6)–water.

RNA fraction	*Specific activity (counts/min/µg RNA)*	
	Expt. No. 2	*Expt. No. 3*
Low-polymeric RNA	9.05	5.0
High-polymeric RNA*	1.17	1.92
RNA-E of phenolic nuclei	20.5	22.1
RNA-N of phenolic nuclei	96.5	71.5

* This fraction apparently represents the RNA of microsomes and nuclear sap.

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Received October 7th, 1959

On the carbohydrate moiety of ovine and human pituitary gonadotropins

Preparations of ovine pituitary follicle-stimulating and interstitial cell-stimulating hormones have previously been reported[1-3] to be glycoproteins. The carbohydrate components in these gonadotropins, however, have hitherto not been identified. We wish to report herein the presence of mannose, galactose, fucose, hexosamine, and sialic acid in these gonadotropins from both human and sheep glands, as detected by colorimetric and chromatographic methods.

Preparations of ICSH and FSH were isolated from fresh sheep or human pituitaries by procedures previously described[4-6]. For the determination of hexosamine, BLIX's modification[7] of the Elson-Morgan procedure was used. Total non-amino sugars were estimated by the procedure of VASSEUR[8], and the values were corrected for the fucose content which was determined by the method of DISCHE AND SHETTLES[9]. Assay for sialic acid was performed according to the procedure of SVENNERHOLM[10] with N-acetylsialic acid* as standard; the values were corrected for the absorption of neutral sugars in Bial's test. Table I summarizes the results of the colorimetric determination of the content of various carbohydrates in preparations of sheep and human gonadotropins.

TABLE I

CARBOHYDRATE CONTENT OF OVINE AND HUMAN PITUITARY FSH AND ICSH PREPARATIONS

Pituitary	*Preparations*	*Carbohydrate contents*, mg/100 mg protein*			
		hexose	*fucose*	*hexosamine*	*sialic acid*
Sheep	FSH	2.30	0.43	3.55	2.44
	ICSH	3.23	0.74	6.26	0.80
Human	FSH	2.10	0.45	2.80	1.89
	ICSH	1.12	0.35	1.95	0.96

* Averaged value from 2 to 6 determinations.

Paper-chromatographic identification of the neutral sugars was performed with the solvent system of MASAMUNE[11], and the chromatograms were developed with aniline phthalate[12] or with $AgNO_3$[13]; the presence of fucose, galactose and mannose was confirmed. Galactose and mannose are present in different ratios in the four gonadotropins tested; in human FSH, the two hexoses were found in an approximately equal ratio; in the sheep preparation, however, galactose was predominant. In the case of ICSH, galactose was by far the predominant hexose in the human material, whereas in the ovine preparations at least twice as much mannose as galactose was present. For human FSH and sheep ICSH, these findings were further supported by comparison of the orcinol color curves with those of the individual hexoses and mixtures thereof.

The existence of both hexosamines in the hydrolysates of ovine ICSH and FSH and of human ICSH was clearly evidenced by paper chromatography[11,14]; so far no

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Abbreviations: FSH, follicle-stimulating hormone; ICSH, interstitial cell-stimulating hormone.

* We wish to thank Prof. G. BLIX of Uppsala for a sample of N-acetylsialic acid.

galactosamine could be detected in human FSH. For the paper chromatographic identification of sialic acid, the solvent system of MARTENSSON *et al.*[15] was used and the spots were visualized with the orcinol reagent[16]. It may be pointed out that the presence of sialic acid in pituitary gonadotropins has not previously been demonstrated. This work was supported in part by a research grant (D-4097) from the U.S. Public Health Service.

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Received November 21st, 1959

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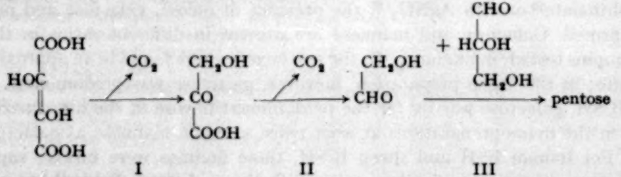
Biochim. Biophys. Acta, 37 (1960) 375–376

Some metabolic pathways of hydroxypyruvate and dihydroxyfumarate leading to the formation of pentose and organic acids in rat liver

As a new pathway for glyconeogenesis, AKABORI *et al.*[1] and UEHARA[2-4] proposed the following sequence of enzymic reactions:



They reported that D-ribose was formed from DHF and D-glyceraldehyde with rabbit-muscle extract, and that Reaction (I) was accelerated by the action of metal ions such as Co^{++}, Mn^{++}, or Mg^{++}. However, it has not been ascertained whether the reaction was actually enzymic or non-enzymic.

Abbreviations: DHF, dihydroxyfumaric acid; HPA, hydroxypyruvic acid; TAS, tartronic acid semialdehyde; TDP, thiamine diphosphate.

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In the present study, it was confirmed that xylulose was formed from DHF, HPA, or TAS, in the presence of DL-glyceraldehyde and purified rat-liver transketolase. The identification of the reaction product was made by paper chromatography[5], the cysteine-carbazole reaction[5,14], and by spectrophotometry of the product of orcinol reaction[5,7]. The spot of xylulose on the paper chromatogram, located with the trichloroacetic acid-orcinol reagent, changed in colour from purple to grey-purple by spraying with aniline-phosphate reagent[5], which is specific for xylulose. Other pentoses were not found in the reaction mixture. Transketolase was prepared according to the procedure of HORECKER *et al.*[9] HPA (lithium salt) was prepared according to the method of DICKENS AND WILLIAMSON[6], and TAS (potassium salt) by spontaneous decarboxylation of DHF at 40°.

Further it was observed that DHF was decarboxylated by an enzyme purified partially by ammonium sulphate and methanol fractionation. A fraction obtained between 30 and 50 % methanol had a specific activity of 15 times that of the acetone-powder extract (Fig. 1). Mn^{++} was required for the enzyme activation, while Mg^{++} was inactive. The reaction products were detected as the 2,4-dinitrophenylhydrazine derivatives by paper chromatography[8,10]. The enzymic system gave a definitely higher amount of the *bis*-2,4-dinitrophenylhydrazone (osazone) as compared with the spontaneous decarboxylation of DHF. Since synthesized TAS was found to form an osazone, while HPA could form only a monohydrazone, it was inferred that the main product of the enzymic reaction was TAS and not HPA, both of which had been considered to be the possible product of enzymic decarboxylation of DHF. The possibility was considered that the CO_2 liberated in the experiment given in Fig. 1 might have been derived not solely from DHF but partly from TAS or HPA. Experiments showed, however, that HPA (30 μmoles) was not decarboxylated at all, either enzymically or non-enzymically under the condition used in the experiments, while TAS (30 μmoles) was decarboxylated spontaneously to some extent, but not at all

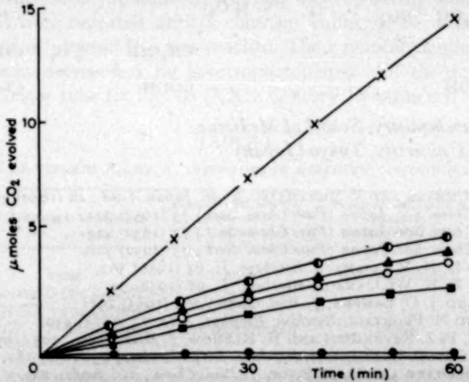
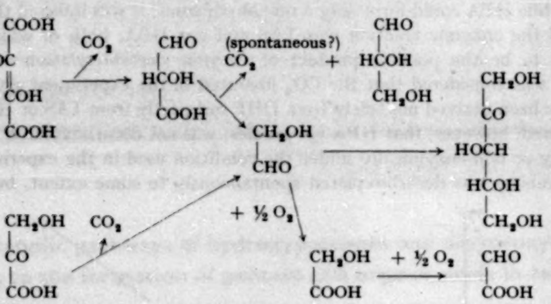


Fig. 1. The enzymic decarboxylation of dihydroxyfumarate. The reaction mixture contained 150 μmoles acetate buffer (pH 5.0); 1 μmole $MnCl_2$; 30 μmoles DHF, or TAS, or HPA; 1.8 mg enzyme protein; total volume was 3.0 ml. Gas phase N_2. Incubated at 30°. ×, complete system; ◐, Mg^{++} instead of Mn^{++}; ▲, with heated enzyme protein; ○, spontaneous decarboxylation of DHF; ■, TAS; ●, HPA. Oxaloacetate was not decarboxylated enzymically under these conditions.

enzymically. The fact that, in the presence of DHF, the enzymic decarboxylation occurred more strongly (about 2 times) than the spontaneous reaction may be taken as an evidence that the decarboxylation observed in the above experiment was due mainly to enzymic decarboxylation of DHF.

Under certain experimental conditions, HPA was found to be decarboxylated enzymically with the formation of glycolaldehyde. This decarboxylation reaction occurred only in the presence of Mg^{++}, while the presence or absence of TDP had no effect. The formation of glycolaldehyde from HPA was confirmed not only from the characteristic absorption spectrum shown by the product of the cysteine–carbazole reaction[14], but also from the following evidence. The enzyme solution which contained the decarboxylation product of HPA was deproteinized with $HClO_4$. After removing the latter by neutralization with KOH, an aliquot of the solution was incubated aerobically with the enzyme solution, "Fraction B", prepared according to the method of KUN *et al.*[11]. The O_2 consumption was observed and it was confirmed that the products were glycolic acid and glyoxylic acid, the former determined by 2,7-dihydroxynaphthalene test and paper chromatography[12,13], and the latter, by paper chromatography of its 2,4-dinitrophenylhydrazone. Based on the observations described above the overall reactions starting from DHF and HPA may be represented as follows:



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Received October 2nd, 1959

Intermediates in the biosynthesis of vitamin B_{12}

Studies performed in our laboratory showed that guanosine diphosphate factor B and factor B phosphate ester are often present in cultures of *Nocardia rugosa*, a vitamin B_{12}-producing microorganism[1–3]. The biological significance of these compounds was discussed in preceding papers[2,3]. Some data which are now available support the opinion that both GDPB and BP are intermediates in the biosynthesis of vitamin B_{12} by *N. rugosa*.

By irradiation of *N. rugosa* with ultraviolet light a mutant strain (959) able to biosynthesize vitamin B_{12} only in a medium supplemented with free factor B, factor B phosphate or guanosine diphosphate factor B was obtained. It seemed of interest to find out whether only the factor B moiety or the whole factor B phosphate moiety belonging to the latter substances is incorporated into vitamin B_{12}. A culture of *N. rugosa*, strain 959, was inoculated into 500-ml flasks each containing 100 ml of growth medium. At the same time $Na_2H^{32}PO_4$ was added to the flasks to give a radioactivity of 1 μC/ml. The medium contained glucose, plant-protein hydrolysate, casein hydrolysate, molasses and mineral salts. The inorganic phosphate content amounted to 1.25 mg P/ml.

The cultures were grown in shaker at 34° for 48 h and then supplemented with factor B or with GDPB* or with BP**. The incubation was carried on for further 72 h. The microorganisms of each flask were collected by centrifuging, then repeatedly washed with phosphate buffer (0.067 *M*, pH 6), and resuspended in 1% NaCN, adjusted to pH 7. The suspensions were autoclaved at 120° for 20 min, the solids were removed by centrifuging and vitamin B_{12} was extracted from the supernatant according to RUDKIN and TAYLOR's method[4]. To remove interfering impurities the vitamin B_{12} contained in the resulting aqueous solution was extracted with phenol. About 4–5 vol. ether were added to the phenol solution, the vitamin B_{12} was re-extracted with water and the aqueous solution was repeatedly washed with ether. These operations were repeated until a constant value of the radioactivity of the aqueous solutions of vitamin B_{12} was reached. The cyanocobalamin content of the final solutions was determined by spectrophotometry and the radioactivity was measured by a Geiger tube for liquids (XXth Century Electronics).

TABLE I

BIOSYNTHESIS OF VITAMIN B_{12} BY *N. rugosa* 959 IN A MEDIUM CONTAINING $Na_2H^{32}PO_4$§

Additions to the medium	*Amount of vitamin B_{12} produced§§ μg/ml*	*Radioactivity in vitamin B_{12} extracted counts/min/μg B_{12}*
none	traces	not determined
factor B (10 μg/ml)	3.9	3.7
GDPB (10 μg/ml)	2.7	0.19
BP (10 μg/ml)	not determined	0.65

§ Specific activity of phosphorus in medium = 115 counts/min/μg P.
§§ Microbiologically determined by *Lactobacillus leichmannii*.

Abbreviations: GDPB, guanosine diphosphate factor B; BP, factor B phosphate ester.

* Isolated from a culture of *N. rugosa* as described in a preceding paper[2].

** Obtained by incubation of GDPB with *Vipera aspis venom*[3].

The identity with vitamin B_{12} of the substance extracted as well as the absence of other factors of the vitamin B_{12} group were checked by paper chromatography.

The data reported in Table I show that the vitamin B_{12} biosynthesized in presence of guanosine diphosphate factor B was only negligibly labeled. More radioactivity was found in the vitamin B_{12} formed in the presence of BP, probably due to small amounts of free factor B present in the BP preparation.

On the basis of these data it is concluded that both the whole molecule of BP and the BP moiety of GDPB are incorporated into vitamin B_{12}. Therefore these two compounds may be considered intermediates in the biosynthesis of vitamin B_{12}.

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Received November 4th, 1959

Biochim. Biophys. Acta, 37 (1960) 379–380

Studies on the mechanism of action of streptomycin. Effect of streptomycin on the excretion of nucleotides by *Escherichia coli*

Previous studies[1] on the mechanism of the antibiotic action of streptomycin on *Escherichia coli* have shown that under certain conditions the lethal action of the antibiotic is inversely related to the inorganic phosphate concentration of the medium over a range of 10^{-2} to 10^{-6} *M*. Since phosphate metabolism appears to be implicated in the bactericidal action of streptomycin, phosphate-balance studies were performed and showed a considerable increase in organic phosphate in the external medium when both phosphate and streptomycin were present. It therefore seemed desirable to isolate and characterize these organic phosphates in order to trace their origin as an aid in understanding the mechanism of action of the antibiotic.

Five-gram (wet wt.) portions of *E. coli* B, sensitive to streptomycin, were incubated for 4 h at 37° under aeration in two 500-ml portions of a medium containing 0.011 *M* lactate, 0.15 *M* NaCl, 0.067 *M* phosphate, pH 7.0. One sample contained 500 μg streptomycin/ml, the other no streptomycin. The cells were centrifuged from the medium at 4° and the organic phosphates present in the media after incubation were separated by the barium-salt procedures of Umbreit *et al.*[2] into water-insoluble

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

and alcohol-insoluble fractions. Amino acids were removed from the alcohol-insoluble fractions by ion-exchange procedures before characterization of the ingredients as reported below.

The ultraviolet-absorption spectra of solutions of the phosphate esters from both the water-insoluble and alcohol-insoluble fractions obtained from media C and D were found to be characteristic of purine and pyrimidine compounds. Pentose was found in all fractions, but no deoxyribose or reducing sugar could be detected. All of the organic phosphate, pentose, and ultraviolet-absorbing materials from all fractions were absorbed on Darco G-60 charcoal, leading to the conclusion that all of the phosphorus in these fractions was nucleotide phosphorus. Treatment of the various fractions with prostatic acid phosphatase gave in each case 100% conversion of organic phosphate to inorganic phosphate, indicating that only mononucleotides were present.

5′-ribonucleoside monophosphates were separated from 2′- or 3′-nucleotides by oxidation of the former by periodate followed by alkaline hydrolysis which dephosphorylates the 5′-nucleotides but will not react with 2′- or 3′-nucleotides[3]. The intact 2′- or 3′-nucleotides can then be separated from the hydrolyzed products of the 5′-nucleotides by precipitation of the former as their barium salts as already described. After hydrolysis in 70% $HClO_4$, the resulting bases associated with each group of nucleotides were quantitatively determined by the method of MARSHAK AND VOGEL[4].

The nucleotides excreted in the presence of phosphate without streptomycin differed in nature and amount from those excreted when both streptomycin and phosphate were present. The results are given in Table I.

The observed increases in extracellular nucleotides may result from (a) specific internal metabolic blocks in their utilization; (b) from degradation of cellular RNA; or (c) from non-specific secondary effects, such as lysis. Evidence against the last possibility may be obtained from the following considerations. Cellular lysis would cause increases in external protein and nucleic acid. Actually the medium containing

TABLE I

EFFECT OF STREPTOMYCIN ON NUCLEOTIDE EXCRETION IN THE PRESENCE OF INORGANIC PHOSPHATE

Medium	*Fraction*	*Bases from nucleotide fractions**					*Total bases***
		Cytosine µmoles	*uracil µmoles*	*hypoxanthine µmoles*	*adenine µmoles*	*guanine µmoles*	
C (no streptomycin)***	5′-nucleotides	6.6	17.6	—§§§	9.4	13.2	46.8
	2′- or 3′-nucleotides	1.1§	2.0§	—§§§	2.3§	3.4§§	8.8
D (+ streptomycin)***	5′-nucleotides	58.0	35.0	28.0	23.0	22.0	166.0
	2′- or 3′-nucleotides§	—§§§	—§§§	—§§§	—§§§	—§§§	—§§§

* Per 100 ml incubation medium.

** The respective total pentoses were 47.0, 9.0, 168 and 0 µmoles.

*** Total organic phosphate was 55.6 µmoles in C, and 168 µmoles in D, per 100 ml incubation mediu.m

§ Determined by base analysis of the 80% alcohol-insoluble barium phosphate esters remaining after oxidation of 5′-nucleotides with metaperiodate.

§§ Determined similarly to § on the water-insoluble barium phosphate esters obtained in the fractionation of the excreted organic phosphate.

§§§ Indicates that none found.

streptomycin contained less external protein than the medium containing no antibiotic, yet contained 3 times as much external organic phosphate. No nucleic acid was found in either medium, nor was any significant loss in turbidity observed during the course of incubation.

The nucleotides excreted in the absence of streptomycin were 85 % 5′-esters and 15 % 2′- or 3′-esters. In the presence of streptomycin no 2′- or 3′-nucleotides were excreted while the excretion of 5′-nucleotides was greatly enhanced. It is also noteworthy that inosinic acid was excreted only in the presence of the antibiotic.

The possibility that the nucleotides excreted into the medium may have arisen from degraded RNA cannot be excluded since about 60 % of the RNA in the cells at the start of incubation (212 μmoles phosphorus) had disappeared after 4 h aeration. This was also reflected by the abnormally low RNA/DNA ratios obtained (less than 2 : 1). Normal ratios in stationary phase cells are about 4 : 1.

It would appear from these data that in the presence of inorganic phosphate, streptomycin causes an enhanced excretion of 5′-nucleotides and prevents the excretion of 2′- or 3′-nucleotides. It is not clear from these results whether streptomycin under these conditions blocks synthesis of RNA from 5′-nucleotides *de novo* or whether degradation of RNA to 5′-nucleotides is enhanced. The loss of sufficient RNA during incubation to account for the excreted nucleotides favors the latter possibility while the finding of excreted inosinic acid only in the presence of streptomycin favors the former. Further work is in progress to aid in clarifying the origin of the excreted 5′-nucleotides.

This work was supported in part by NIH Grant E-1308, from the National Institute of Allergy and Infectious Diseases, Public Health Service.

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Received October 12th, 1959

Biochim. Biophys. Acta, 37 (1960) 380–382

Formation of acetone and acetoacetate from cholesterol by rat and mouse liver mitochondria

Previous studies have indicated that the terminal methyl groups of the cholesterol side chain are oxidized to CO_2 by suitably fortified preparations of rat[1] and mouse[2] liver mitochondria. This cholesterol oxidase activity is believed to be associated with the biogenesis of bile acids from cholesterol—a process requiring removal of the terminal isopropyl group from the cholesterol side chain, as well as nuclear saturation and hydroxylation.

Abbreviations: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide.

We have detected the presence of radioactive acetone amongst the products of incubating [26-^{14}C]cholesterol with rat and mouse liver mitochondria. Distillation of the incubates at an alkaline pH yielded a steam-volatile substance which (a) formed a radioactive 2,4-dinitrophenylhydrazone and *p*-toluenesulphonylhydrazone, chromatographically identical with those formed from acetone and (b) form a radioactive precipitate on refluxing with $HgSO_4$ in H_2SO_4 (Déniges reagent). The yield of radioactive acetone was increased by extracting sucrose-washed liver mitochondria with 1.15 % (*w/v*) KCl and conducting incubations in the absence of glutathione and with the addition of Zn^{++} (10^{-4} *M*). Under these conditions, the yields of radioactive acetone and acetoacetate from sodium [1-^{14}C]octanoate are increased, with corresponding reduction in the yield of $^{14}CO_2$.

Cholesterol labeled *inter alia* at C_{25} was prepared by incubating rat liver slices with sodium [1-^{14}C]acetate[3]. Liver mitochondria from male Wistar rats and Swiss Hygienic Mice were isolated as previously described[1], further extracted with isotonic KCl, re-isolated and finally suspended in 10 % (*w/v*) sucrose for addition to the incubations. Each incubation contained penicillin G (1,000 Oxford units of sodium salt), streptomycin sulphate (0.5 mg), ATP (12.5 mg), DPN (2.5 mg), $Mg(NO_3)_2 \cdot 6H_2O$ (5 mg), and $ZnSO_4 \cdot 7H_2O$ (10^{-4} *M*) in a final volume of 6.3 ml. Cholesterol was emulsified[1] in Tween 20 and 5 ml 0.125 *M* tris(hydroxymethyl)aminomethane–HCl buffer, pH 8.5. The cholesterol was freshly purified by chromatography over alumina before use. Incubations were shaken for periods between 6 and 16 h at 37°.

Acetone was isolated by steam distillation of the incubates after addition of water (40 ml) and sodium acetoacetate (1.5 mg) without adjustment of the pH. (At acid pH's, the steam volatility of cholesterol is somewhat enhanced.) The distillate (10 ml) was refluxed for 30 min with 1.0 ml conc. H_2SO_4 and 4.5 ml 15 % (*w/v*) $HgSO_4$ in dil. H_2SO_4 (minimum amount to prevent hydrolysis and precipitation of mercuric oxide). Acetone was recovered from the Déniges precipitates thus formed, after their dissolution in 2 ml 2 *N* HCl and addition of water, by redistillation. Iodoform was prepared from these distillates or, preferably, from the first (mercury-free) distillate from the incubation mixtures. Attempts to increase the yield of acetone and acetoacetate by oxidizing the sometimes considerable amounts of radioactivity present as *β*-hydroxybutyrate also resulted in some chemical degradation of the unmetabolized cholesterol to acetone. Incubates with boiled mitochondria were always worked up as controls. Sodium [1-^{14}C]octanoate was incubated with a portion of each of the liver preparations in order to demonstrate the formation of acetoacetate by known reactions in this system and to indicate the efficiency and validity of the recovery and degradative techniques used.

Degradation of the acetone by the iodoform reaction indicated that the methyl groups were labeled when the acetone was derived from [26-^{14}C]cholesterol. Acetone derived from incubations with [25-^{14}C]cholesterol or sodium[1-^{14}C]octanoate was not labeled in the methyl group.

Acetoacetate, present in the original incubation mixtures, was degraded as follows. Respiratory CO_2 was removed by bubbling a stream of alkali-washed air through the incubation mixture, after acidification to pH 4.5 with 0.25 *M* succinic acid. Aniline succinate and carrier sodium acetoacetate (3 mg) were then added and the CO_2, liberated by the aniline-catalyzed decarboxylation of acetoacetic acid, was trapped in NaOH and precipitated with $BaCl_2$. Incubations with [26-^{14}C]cholesterol and

[25-^{14}C]cholesterol gave no $^{14}CO_2$ under these latter conditions, whilst those with [1-^{14}C]octanoate yielded $^{14}CO_2$ (from the carboxyl group of acetoacetic acid).

Table I summarizes results which suggest that current theories involving the mode of degradation of the cholesterol side chain accompanying bile acid formation[4–7] *viz*, ω-oxidation succeeded by β-oxidation and cleavage of acetyl coenzyme A, require modification. If the results obtained with [25-^{14}C]cholesterol are valid (unequal labeling of the carbonyl groups in the resultant acetoacetate), then the following alternatives should be considered:

(a) loss of one carbon atom followed by cleavage of a two-carbon fragment which is probably not acetyl coenzyme A,

(b) initial cleavage between carbon atoms 24 and 25 to yield acetone directly or indirectly.

TABLE I

FORMATION OF ACETONE BY RAT LIVER MITOCHONDRIA

Substrate	*Radioactivity (counts/min) of*		
	mercury-acetone (Denigès ppt)*	*iodoform*	*CO_2 from acetoacetate*
Sodium [1-^{14}C]octanoate			
1st expt. (14,500 counts/min)	2560 (36)	14	2320
2nd expt. (23,500 counts/min)	2840 (41)	0	1918
[26-^{14}C]Cholesterol			
1st expt. (37,500 counts/min)	912 (232)	150	28
2nd expt. (34,100 counts/min)	428 (36)	168	12
[25-^{14}C]Cholesterol			
1st expt. (6,400 counts/min)	202 (47)	0	12
2nd expt. (2,040 counts/min)	132 (14)	0	4

* Values in parentheses represent radioactivity from incubations with boiled mitochondria.

This work was supported by grants from the National Institutes of Health, Bethesda, Md., U.S.A.

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Received November 23rd, 1959

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Lombricine phosphoryltransferase in earthworms

The discovery of lombricine[1] (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate) in earthworms, together with the subsequent finding[2,3] that this substance is the phosphodiester of guanidinoethanol and D-serine, was followed by its chemical synthesis[3] and isolation[4,5] in preparative amounts. These results together with the isolation[6] of a labile N-phosphorylated mono-substituted guanidine containing a labile P and a guanidine residue in equimolar proportion suggested that N-phosphoryl lombricine, the synthesis of which has been accomplished[3], is the "phosphagen" in the earthworm. Proof of such a suggestion must lie in the adequate demonstration of an enzyme capable of catalysing the reaction:

$$\text{Lombricine} + \text{ATP} \rightleftharpoons \text{N-phosphoryl lombricine} + \text{ADP}$$

Such an enzyme has been found in extracts of the muscles of the giant earthworm (*Megascolides cameroni*) and some of its properties are herein described.

The enzyme preparation was a dialysed ammonium sulphate fraction (50–90% saturation) of a worm-muscle extract in 0.5 *M* KCl buffered with N-ethylmorpholine at pH 7.2.

The ability of the enzyme to catalyse the transfer of a phosphoryl group as expressed in the reaction above has been tested under varying conditions by determination of the free guanidine[7] before and after reaction. In N-ethylmorpholine buffer (0.1 *M*), and in the presence of Mg^{++} (0.0015 *M*) at 30° the optimum activity was found to be at pH 8.4.

The enzyme was activated by Mg^{++}, Co^{++} and Mn^{++}, but no activation occurred in the presence of Ca^{++} or Sr^{++}. In the absence of added Mg^{++} there was only negligible activity and there was no activity if ATP was omitted. The enzyme was inhibited by *p*-chloromercuribenzoate ($1 \cdot 10^{-5}$ *M*).

Under optimal conditions with respect to pH, Mg^{++}-concentration and in the presence of a 5-fold excess of ATP, 80% phosphorylation of added lombricine was achieved in 10 min at 30°. Quantitative recoveries of lombricine from the phosphorylated product resulting from the incubation have been obtained by hydrolysis for 9 min in 0.01 *N* HCl at 65°.

Preliminary investigations of the substrate specificity of the enzyme in so far as the guanidine base is concerned have revealed a surprising lack of stereospecificity in that L-lombricine was phosphorylated more rapidly than the naturally occurring D-isomer (Fig. 1).

A number of mono- and di-substituted guanidino compounds have also been tested for their ability to act as substrates under conditions similar to those in which L- or D-lombricine was rapidly phosphorylated. D- and L-arginine, guanidinoethylphosphate, guanidinoethanol, ethylguanidine, creatine, guanidinoacetic acid, α-guanidinopropionic acid, β-guanidinopropionic acid and γ-guanidinobutyric acid were incapable of acting as substrates, but guanidinoethanesulphonic acid (taurocyamine) was phosphorylated to yield N-phosphoryltaurocyamine (Fig. 1). This result was unexpected particularly as both guanidinoethylphosphate and β-guanidinopropionic acid were inactive as substrates. At present it is not known whether this result was due to a lack of specificity of lombricine phosphoryltransferase or to the

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Abbreviations: ATP, ADP, adenosine tri- and di-phosphate.

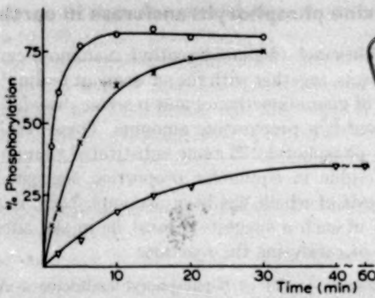

Fig. 1. Rate of phosphorylation of D- and L-lombricine and taurocyamine by ATP in the presence of earthworm muscle extract. The incubation mixture (1 ml) contained: D-lombricine (×), L-lombricine (○) or taurocyamine (△), $5 \cdot 10^{-4}$ *M*; cysteine, $1 \cdot 10^{-4}$ *M*; $MgCl_2$, $1.5 \cdot 10^{-3}$ *M*; ATP, $5 \cdot 10^{-3}$ *M*; N-ethylmorpholine buffer, pH 8.2, 0.1 *M*; and 0.1 ml of the muscle extract, equivalent to 12.5 mg muscle. Temperature, 30°.

presence of taurocyamine phosphoryltransferase in the enzyme preparation. The latter possibility would appear unlikely in view of the complete absence of taurocyamine in earthworm tissue.

These results leave no doubt as to the function of lombricine as a phosphagen precursor as was originally suggested by THOAI *et al*[6].

A detailed study of the properties of the enzyme will be published shortly.

Our thanks are due to Dr. D. I. MAGRATH and Mrs. I. M. BEATTY for gifts of L-lombricine and guanidinoethylphosphate, and to the late Dr. J. GREENSTEIN for a gift of D-arginine.

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Received November 17th, 1959

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Stimulating effect of thyroxine in contraction of rat liver mitochondria

ATP has been found to be capable of contracting rat liver mitochondria with extrusion of water following swelling induced by a wide variety of agents, which include thyroxine, Ca^{++}, inorganic phosphate, hypotonicity, *p*-chloromercuribenzoate, U factor, and oleate[1-4]. Such contraction is usually maximal when ATP is supplemented by Mg^{++} (or Mn^{++}) and small amounts of bovine serum albumin; however a wide variety of other substances tested have failed to stimulate the action of ATP[2,3].

It has now been found that under certain circumstances L-thyroxine greatly potentiates the contraction of swollen rat liver mitochondria by ATP, producing more complete and more sustained contraction. The effect is best demonstrated when rat liver mitochondria are induced to swell in a hypotonic medium or in a phosphate-containing medium, in the absence of added thyroxine. The typical experiment in Fig. 1 shows the rapid swelling produced in a hypotonic medium of 0.01 *M* tris-(hydroxymethyl)aminomethane–HCl buffer, pH 7.4. At the point shown, addition of ATP and adjuvants such as Mg^{++}, serum albumin and thyroxine were made. It is seen that addition of 0.005 *M* ATP alone produced only a slight and transistory contraction. However, if $1 \cdot 10^{-5}$ *M* thyroxine was added with the ATP, then a substantial contraction of the mitochondria ensued, which was sustained for a long period. Addition of L-thyroxine without ATP failed to induce contraction. Mg^{++} could replace L-thyroxine in potentiating the action of ATP, but was not as effective in maintaining the contraction. The combinations of ATP, Mg^{++}, and serum albumin or ATP, thyroxine and serum albumin produced maximal contraction. Similar observations on the stimulating effect of thyroxine on ATP-induced contraction were made on rat liver mitochondria swollen in a phosphate-containing medium. The stimulating action of thyroxine was established not only by following absorbancy changes as shown but also by direct measurement of changes in water content of the mito-

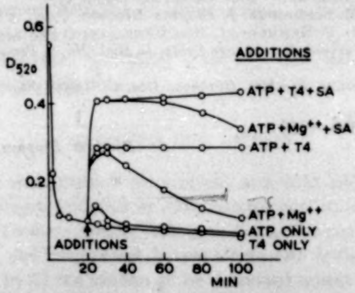

Fig. 1. Stimulation of mitochondrial contraction by L-thyroxine. Fresh washed mitochondria derived from 50 mg rat liver were added to tubes containing 5.0 ml 0.01 *M* tris(hydroxymethyl) aminomethane–HCl buffer, pH 7.4 at 20°. Absorbancy changes at 520 mμ were observed. After swelling had taken place, ATP, thyroxine and other agents were added at the point shown by the arrow, to yield the following final concentrations: ATP (0.005 *M*), L-thyroxine ($1 \cdot 10^{-5}$ *M*), $MgCl_2$ (0.003 *M*), crystallized bovine serum albumin (2 mg/ml).

Abbreviation: ATP, adenosine triphosphate.

chondria[2,3]. The contracting effect of L-thyroxine is maximum at pH 7.4 and at low concentrations of mitochondria.

The potentiating action of L-thyroxine in stimulating mitochondrial contraction by ATP is rather unexpected, since L-thyroxine is itself a very potent agent in causing mitochondrial swelling[1,4,5]. Other mitochondrial swelling agents such as inorganic phosphate[6], Ca^{++}, reduced glutathione[7], and oleate[8] do not stimulate mitochondrial contraction under the same conditions as does L-thyroxine, indicating that this effect of L-thyroxine is rather unique and specific. A basis for the stimulating action of thyroxine may be found in the observation that the effect can also be produced in part by Mg^{++}; L-thyroxine is able to bind certain metal ions[1,4,9] and may in this case be able to bind an endogenous (bivalent) metal ion in the mitochondrion, which is inhibitory to ATP-induced contraction and whose action can also be opposed by Mg^{++}. However, L-thyroxine is not able to replace Mg^{++} in the medium in all instances of ATP-induced contraction.

These experiments thus show that L-thyroxine may promote not only the swelling of mitochondria but also the ATP-linked contraction; it appears to be relatively unique in this property.

This investigation was supported by grants from the National Science Foundation, the Nutrition Foundation, Inc., and the Whitehall Foundation.

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Received November 23rd, 1959

THE PEPTIC ACTIVATION OF ACETYLTRYPSINOGEN: PHYSICOCHEMICAL PROPERTIES OF THE ACTIVE DERIVATIVE*

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(Received January 23rd, 1959)

SUMMARY

The active derivative (ATd) produced by the peptic degradation of acetyltrypsinogen had the following physicochemical properties:

1. A molecular weight of 5500–6500 based on its amino acid composition and reaction with $DF^{32}P$.
2. An N-terminal residue of phenylalanine.
3. A sedimentation constant of 0.95S.

ATd appears to possess a broader specificity than trypsin as evidenced by its ability to hydrolyze tyrosine methyl ester and oxidized insulin to a much greater degree than trypsin. The implication of these findings in relation to the structural and catalytic features of trypsin is discussed.

INTRODUCTION

The purification and isolation of an active derivative produced by the peptic activation of acetyltrypsinogen has been reported in previous communications[1,2]. The present paper is concerned with the physicochemical characterization of this active derivative. Presented herein are data relative to its sedimentation behavior, amino acid composition, substrate specificity, and reaction with diisopropylphosphofluoridate and *p*-nitrophenylacetate.

MATERIALS AND METHODS

The active derivative was purified by precipitation with $(NH_4)_2SO_4$ from a peptic digest of acetyltrypsinogen followed by chromatography on a column (1.2 × 40 cm) of DEAE-cellulose[2]. Fractions corresponding to the active peak (see Fig. 1f of ref. (2)) were pooled, dialyzed, and lyophilized. Preparations such as these, henceforth referred to as ATd, were used in all the studies to be described unless stated otherwise.

Trypsinogen (1 × crystallized, 50% $MgSO_4$), trypsin (2 × crystallized, salt-free), chymotrypsin (3 × crystallized), and pepsin (2 × crystallized) were all purchased from Worthington Biochemical Corporation, Freehold, N.J. For most of the comparative experiments involving ATd and trypsin, the latter was prepared from the same lot of trypsinogen by autocatalytic activation in the conventional manner[3]. Acetyltrypsin was prepared in the same manner as acetyltrypsinogen[1]. Samples of crystalline insulin, obtained from Lilly Research Laboratories, Indianapolis, Ind., courtesy of Dr. O. K. BEHRENS, or the Boot Pure Products Co., Nottingham, England, were oxidized according to the procedure of SANGER[4]. Carbobenzoxy-L-glutamyl-L-tyrosine, benzoyl-L-arginine ethyl ester, and benzoyl-L-argininamide were purchased from the Mann Research Laboratories, N.Y. L-Tyrosine methyl ester was a gift from Dr. S. KIRKWOOD of this Department. ^{32}P-labeled diisopropylphosphofluoridate (DF^{32}P) was purchased from the Manning Research Laboratories, Waltham, Mass., and was stated to be 96 % pure*. The *p*-nitrophenylacetate (NPA) was prepared by Dr. H. FUNATSU at the Carlsberg Laboratorium, Copenhagen, Denmark.

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Acid hydrolysates of ATd, prepared by refluxing 5–6 mg in 1 ml constant boiling HCl (5.7 *N*) in a sealed tube for 18–20 h, were qualitatively examined for amino acids by one-dimensional paper chromatography using butanol–acetic acid–water[5] or the two-dimensional solvent systems of REDFIELD[6]. A quantitative estimation of the amino acids was performed by ion-exchange chromatography on a column of Dowex-50-4X in accordance with the directions of MOORE AND STEIN[7]. In addition to these techniques, several other methods were employed for the determination of histidine including the colorimetric methods of MCPHERSON[8] and COWGILL[9], microbiological assay using *Leuconostoc mesenteroides* P-60[10], and chromatography on the cation-exchange resin IRC-50[11].

N-terminal group analyses using dinitrofluorobenzene were performed as described by FRAENKEL-CONRAT *et al.*[12]. Corrections for losses of dinitrophenyl-(DNP-) amino acids during hydrolysis were made using the factors given by PORTER AND SANGER[13]. Chromatographic losses were assumed to be 10 %[12].

Esterase activity on benzoyl-arginine ethyl ester or tyrosine ethyl ester was measured by the spectrophotometric procedure of SCHWERT AND TAKENAKA[14] at 253 mμ and 233.5 mμ respectively. Amidase activity on benzoyl-argininamide was determined by the diffusion technique of BRAGANCA *et al.*[15] in which the liberated NH_3 was absorbed by acid in the center well of a Warburg flask and measured colorimetrically with NESSLER's reagent[16]. The hydrolysis of carbobenzoxy-glutamyl-tyrosine was followed by the increase in free amino groups as measured with ninhydrin[17]. This assay was conducted at pH 4.0 (0.1 *M* acetate buffer) or pH 6.9 (0.067 *M* phosphate buffer) using 2 ml of a solution containing 0.1 mmole of the substrate and 0.2 ml of enzyme solution. The hydrolysis of oxidized insulin was likewise measured by the increase in amino groups in a system composed of 5 ml of 0.067 *M* phosphate buffer, pH 6.5, containing 1 μmole of the substrate and to which was added 0.1 ml of a solution containing 125–130 μg of enzyme protein. All of the above assays were performed at 37° and pH 8.0 (0.01 *M* borate buffer) unless specified otherwise.

The reaction of ATd with DF^{32}P was performed by treating a solution of unchromatographed ATd containing 5 to 10 mg protein dissolved in 0.1 *M* phosphate buffer, pH 7.0, or 0.1 *M* borate buffer, pH 8.0, with 1 ml of 0.1 *M* DF^{32}P in isopropanol, and adjusting the final volume to 10 ml with buffer. When the enzymic activity had been reduced to a negligible value, the excess DF^{32}P was removed by dialysis (18/32 Visking casing) until the radioactivity of the dialysate was equal to the background rate*. Measured aliquots (0.2 to 0.5 ml) of the solution were applied to planchets, whose bottoms were fitted with circles of filter paper. After adding 0.6 ml of 1 *M* BaCl$_2$ made up in 0.02 *M* NaOH, the solutions were evaporated to dryness under an infra-red lamp. Samples were then counted in the usual fashion under a Geiger tube. A sample of the original solution of DF^{32}P was counted at the same time.

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* The use of DF^{32}P was authorized under an Atomic Energy Commission license issued to Dr. M. O. SCHULTZE to whom we are indebted for his assistance with this phase of the work.

In experiments with NPA 9 ml of 0.067 *M* phosphate buffer, pH 7.6, containing 0.5 to 1.0 mg of enzyme was treated with 1 ml of $5.5 \cdot 10^{-3}$ *M* NPA in absolute ethanol. The formation of *p*-nitrophenol was measured spectrophotometrically at 400 mμ. A control system from which the enzyme had been omitted was included to correct for the spontaneous hydrolysis of NPA.

The sedimentation constant of ATd was measured in a Spinco, Model E, ultracentrifuge operated at a maximum speed of 59,780 rev./min. The slow rate of sedimentation that was observed with ATd necessitated the use of a synthetic boundary cell. We are indebted to Dr. D. R. BRIGGS for running this analysis.

The protein concentrations of solutions of ATd were calculated either on the basis of N × 6.25 where N was determined by a micro-Kjeldahl method[16] or by multiplying the absorbance at 280 mμ by a factor of 0.6. The equivalence of these two methods was established experimentally.

RESULTS

Sedimentation studies. In Fig. 1 are presented a series of photographs which show the sedimentation characteristics of ATd. This preparation was apparently homogeneous, as indicated by a single peak which had a sedimentation constant ($S_{20,w}$) of 0.95S. It is particularly noteworthy that there was no indication of the presence of heavier components.

Amino acid composition. Qualitative analysis of an acid hydrolysate of ATd by

Fig. 1. The sedimentation behavior of 0.3 % ATd in 0.1 *M* NaCl as a function of time. Photographs from right to left were taken after 32, 64 and 96 min and at a bar angle of 50°. Temperatures: initial, 24.1°; final, 25.8°. Speed: 59,780 rev./min.

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* Diisopropylphosphoryl-ATd was prepared in essentially the same manner using non-labeled DFP. After dialysis to remove excess DFP, the protein solution was lyophilized.

TABLE I

AMINO ACID COMPOSITION OF THE ACTIVE DERIVATIVE

*Amino acid**	*Weight recovery*		*Molar ratio***		*Nitrogen % of total*
	g/100 g protein	*moles/1000 g protein*	*calculated*	*assumed*	
Aspartic acid	11.0	0.83	5.4	5	7.2
Threonine	6.8	0.57	3.8	4	5.0
Serine	10.8	1.03	6.8	7	9.0
Proline	4.2	0.37	2.4	2	3.2
Glutamic acid	5.2	0.35	2.3	2	3.1
Glycine	21.4	2.85	18.8	19	25.0
Alanine	8.1	0.91	6.0	6	7.9
Valine	5.5	0.47	3.1	3	4.1
Cystine	2.6	0.11	0.7	1	1.9
Leucine Isoleucine	9.1	0.70	4.6	5	6.1
Tyrosine	0.4	0.02	0.1	—	0.2
Phenylalanine	2.5	0.15	1.0	1	1.4
Lysine	12.8	0.88	5.8	6	15.3
Histidine	0.6	0.04	0.2	—	1.0
Arginine	0	0	0	0	0
Total	101.0			61	90.4

* Tryptophan and NH_3 have not been included.
** Phenylalanine taken as 1.0.
*** Since these two amino acids were not completely resolved, the values shown are subject to error.

one or two dimensional paper chromatography revealed the presence of the following amino acids: lysine, proline, aspartic and glutamic acids, serine, threonine, leucine and/or isoleucine, alanine, valine, glycine, and possibly phenylalanine. The presence of tyrosine could not be established with certainty because of its overlapping with valine with the solvent systems employed here[5,6].

The results obtained by ion-exchange chromatography on Dowex-50 are presented in Table I. The molar ratios of the amino acids as shown in the last column were calculated on the assumption that only one phenylalanine residue was present and that it occupied the N-terminus of the molecule (see "N-terminal groups" below). No other phenylalanine residue was deemed to be present in the molecule since previous studies[1,2] had shown that all of the peptide bonds containing the aromatic residues of the original acetyltrypsinogen molecule had been cleaved by pepsin; thus, phenylalanine and tyrosine were present only as N-terminal groups in the final digest from which ATd had been isolated. The molecular weight of ATd based on the amino acid composition shown in Table I is 6650. Since tryptophan was not determined and the value for cystine may be unreliable, this molecular weight must be considered only as an approximation of the true value.

Specific colorimetric tests for histidine[8,9] failed to reveal more than traces of this amino acid in acid hydrolysates of ATd. The results of various experiments to establish with more certainty the histidine content of the active derivative are summarized in Table II. Values for histidine ranged from 0.15 to 0.46 mole/mole ATd depending on the preparation employed. The expected three residues of histidine reported to be present in trypsinogen[18] were obtained by microbiological assay. A

TABLE II

THE HISTIDINE CONTENT OF PREPARATIONS OF THE ACTIVE DERIVATIVE (ATd), TRYPSINOGEN, AND THE PEPTIC DIGEST OF ACETYLTRYPSINOGEN

Material analyzed	*Method employed**	*Histidine content*** *moles/mole protein*	*Specific activity****
ATd, preparation A	Chromatography on Dowex-50 (7)	0.21; 0.23 (0.22)	6.5
ATd, preparation B	Chromatography on IRC-50 (11)	0.44; 0.47 (0.46)	2.1
ATd, preparation C	Microbiological (10)	0.40; 0.44 (0.42)	1.9
ATd, preparation D	Microbiological§§	0.15	5.7
Trypsinogen	Microbiological (10)	2.80; 3.04; 2.75 (2.86)	0
Peptic Digest of acetyl trypsinogen§	Microbiological (10)	2.96; 3.08 (3.02)	0.2

* Literature references are given in parentheses.

** These values are based on molecular weights of 6500 and 24,000 for ATd and trypsinogen respectively. Figures in parentheses are the averages of the experimental values.

*** Expressed as Δ O.D. 253/min/mg protein using benzoyl-arginine ethyl ester as the substrate[14]. The specific activity of ATd varied somewhat from preparation to preparation with different lots of trypsinogen.

§ Unfractionated mixture prior to the isolation of ATd. For further details see ref. (2). Since the molar ratio of acetyltrypsinogen to pepsin was 50 to 1, the histidine contributed by pepsin was considered negligible.

§§ The authors are indebted to Dr. MILTON SILVERMAN, National Institutes of Health, Bethesda, Md. (U.S.A.) for this particular assay.

similar value was obtained for the peptic digest of acetyltrypsinogen from which ATd had not been removed.

N-terminal groups. Preliminary experiments had indicated that either phenylalanine or tyrosine constituted the N-terminus of ATd. One dimensional chromatography employing *t*-amylalcohol and phthalate buffer as the solvent system[18] did not satisfactorily resolve the DNP-derivatives of these two amino acids. Chromatography in a second dimension using 1.5 *M* phosphate buffer[19], however, revealed that DNP-phenylalanine was the major DNP-amino acid in the ethereal extract of the DNP-protein hydrolysate. Traces of other DNP-amino acids, namely glutamic acid, alanine, and threonine, were occasionally noted in some preparations of ATd.

Quantitative estimation of the DNP-amino acids by elution of the spots and measuring the absorbance at 360 mμ gave an uncorrected value of 0.61 moles of DNP-phenylalanine/6500 g protein. Applying a 30% and 10% correction for losses incurred during hydrolysis[13] and chromatography[18] respectively brings this value to 0.95 moles. Other DNP-amino acids, when detected, corresponded to less that 0.08 moles per 6500 g of protein.

Reaction with DF^{32}P. In a typical experiment involving the treatment of ATd with DF^{32}P, the labeled protein had $0.85 \cdot 10^6$ counts/min/mg protein. The DF^{32}P used for labelling the protein had an activity of $4.66 \cdot 10^9$ counts/min/mmole P. From these data it was calculated that 1 mole of P had been incorporated into 5500 g of protein. Similar experiments with trypsin indicated an incorporation of 1 mole P/20,000 g protein.

Reaction with NPA. In Fig. 2 is plotted the release of *p*-nitrophenol when NPA was incubated with ATd. Similar experiments with trypsin, acetyltrypsin, and diisopropylphosphoryl-ATd are shown in the same graph for purposes of comparison. Although ATd significantly enhanced the hydrolysis of NPA, there was no rapid initial release of *p*-nitrophenol as was observed with trypsin and acetyltrypsin. In the last two cases, the intercept obtained by extra polating the linear hydrolysis to zero time corresponded to 0.9 moles and 0.8 moles of *p*-nitrophenol per mole of trypsin and acetyltrypsin respectively. Diisopropylphosphoryl-ATd did not catalyze the hydrolysis of NPA over and above that which could be attributed to spontaneous hydrolysis.

TABLE III

SUBSTRATE SPECIFICITY OF ATd COMPARED TO TRYPSIN

Substrate	*Specific activity*			
	Units	*ATd*	*Trypsin*	*Ratio ATd/Trypsin*
Benzoyl-Arginine ethyl ester	Δ O.D. 253/min/mg protein	6.5***	13.0	0.5
Benzoyl-Argininamide	μmoles NH_3/min/mg protein	0.21	0.61	0.3
Tyrosine methyl ester*	Δ O.D. 233.5/min/mg protein	0.13	0.005	25
Carbobenzoxy-Glutamyltyrosine	moles NH_3/mole substrate	0	0	—
Oxidized insulin**	moles NH_3/mole substrate	7–8	2	3.5–4.0

* The specific activity of chymotrypsin was 2.7 from which it may be calculated that the chymotryptic activity of ATd and trypsin was equivalent to 5% and 0.2% chymotrypsin respectively.

** Based on the data shown in Fig. 3.

*** Specific activity of preparation A (Table II) is used here.

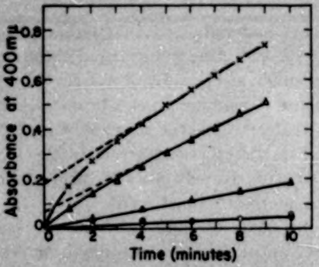

Fig. 2. The hydrolysis of *p*-nitrophenylacetate (NPA) as measured by the release of *p*-nitrophenol. ○—○, spontaneous hydrolysis; ●—●, diisopropylphosphoryl-ATd (0.50 mg); ▲—▲, ATd (0.42 mg); △—△, acetyltrypsin (0.35 mg); and ×—×, trypsin (0.62 mg). Temperature of all systems maintained at 25°.

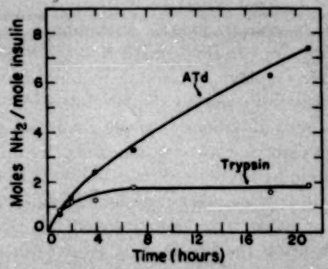

Fig. 3. Hydrolysis of oxidized insulin by ATd and trypsin.

Specificity studies. The results of the action of ATd and trypsin on various substrates are summarized in Table III. The failure of ATd to catalyze the hydrolysis of carbobenzoxy-glutamyltyrosine may be taken to indicate the absence of any contamination with pepsin. The esterase and amidase activities of ATd, as measured on the ethyl ester and amide of benzoyl-arginine, were about one-half and one-third that of trypsin respectively. With tyrosine methyl ester as a substrate, however, the esterase activity of ATd was considerably greater (25 times) than that of trypsin. Further confirmation of the ability of ATd to catalyze the hydrolysis of peptide bonds which are generally considered resistant to the action of trypsin was derived from experiments in which oxidized insulin was employed as a substrate (Fig. 3). In agreement with the findings of SANGER AND TUPPY[21], trypsin cleaved only two peptide bonds, presumably involving the carboxyl groups of the one arginine and one lysine residue known to be present in insulin. ATd, on the other hand, had hydrolyzed almost 8 peptide bonds by the end of 21 h and, judging from the curve in Fig. 3, the hydrolysis was still proceeding at this time.

In an attempt to delineate the specific peptide linkages cleaved during the hydrolysis of oxidized insulin, the 21-h digests shown in Fig. 3 were analyzed for N-terminal groups in the usual manner. After treatment with dinitrofluorobenzene, subsequent acidification yielded a yellow precipitate which was subjected to acid hydrolysis. Both the supernatant and the acid hydrolysate of the precipitate were examined for DNP-amino acids. In the case of trypsin, DNP-alanine was found in the supernatant, and DNP-glycine and -phenylalanine were noted in the hydrolysate of the precipitated protein. These results are what one would expect based on the known amino acid sequence of insulin and the adherence of trypsin to its specificity requirements. In addition to DNP-alanine, the supernatant of the ATd digest also yielded DNP-phenylalanine, -glycine, and -tyrosine while the precipitated protein gave the same DNP-amino acids as were obtained with the precipitate of the tryptic digest. Although conformity with the specificities of chymotrypsin as well as trypsin would account for the results obtained with ATd[21], a more detailed study of the structure of the peptides resulting from the digestion of oxidized insulin by ATd is required before the specificity of ATd can be unequivocally defined.

A summary of some of the interesting points of difference between ATd and trypsin is presented in Table IV.

TABLE IV

A COMPARISON OF THE PROPERTIES OF THE ACTIVE DERIVATIVE (ATd) AND TRYPSIN

Property	*Trypsin**	*ATd*
No. of amino acid residue	195	61
Sedimentation constant	2.4	0.95
Molecular weight	24,000	5500–6500
N-terminal residue	Isoleucine	Phenylalanine
Solubility in 3.6 % TCA**	Insoluble	Soluble
Relative activity***	100	50

* Most of the data pertaining to the properties of trypsin have been taken from review paper by GREEN AND NEURATH[18].

** TCA = trichloroacetic acid. See ref. (1) and (2) for details.

*** Specific activity of trypsin on benzoyl-arginine ethyl ester taken as 100.

DISCUSSION

Preparations of the active derivative used in these studies can be considered virtually homogeneous from the standpoint of sedimentation behavior and N-terminal group analysis. It is true that, in addition to DNP-phenylalanine, which was consistently observed as the major DNP-amino acid, traces of other DNP-amino acids were sometimes encountered pointing to the presence of small amounts of contaminating peptides. The difficulty of removing contaminating peptides from the active site of crystalline samples of chymotrypsin[22] and trypsin[23] has been recognized and a similar situation may obtain in the case of ATd.

From the evidence available, it is believed that the molecular weight of ATd is in the neighborhood of 6000. This conclusion is based upon (1) the amino acid composition of ATd where a molecular weight of 6650 is obtained if, as it appears reasonable to assume, one phenylalanine residue is present as the N-terminus of the molecule, (2) the incorporation of 1 mole P/5500 g ATd when the latter is inactivated by $DF^{32}P$, and (3) the recovery of nearly a stoichiometric amount (0.95 moles) of DNP-phenylalanine/6500 g dinitrophenylated ATd. The observed sedimentation constant of ATd, 0.95 S, would seem reasonably consistent with a molecular weight of 5500–6500. For comparison, it may be noted that recent measurements[24] of the sedimentation constant of insulin (Mol. wt. = 6000), under conditions favoring dissociation, indicate a value of about 1.0 S.

From the studies on the specificity of ATd on various substrates, it would appear that ATd possesses a broader specificity than trypsin itself. This was evident from experiments in which ATd was more active than trypsin on tyrosine methyl ester and oxidized insulin. That this non-specific cleavage by ATd may have been due to contamination with chymotrypsin appears unlikely from the following considerations:

1. The chymotryptic activity of ATd (equivalent to 5% chymotrypsin), as measured on tyrosine methyl ester, was many times that normally associated with trypsin. Most commercial samples of crystalline trypsin, including that obtained by autocatalytic activation of the same lot of trypsinogen used for preparing ATd, were found to have an activity equivalent to about 0.2% chymotrypsin. Thus, the chymotryptic activity of ATd was much more than could be expected even by assuming that all of the chymotrypsin present in the original trypsinogen was preferentially concentrated in ATd.

2. Crystalline chymotrypsinogen, when acetylated and digested with pepsin under the same conditions as trypsinogen, failed to yield a derivative which was active on tyrosine methyl ester.

It has been shown that in several different esterases, including chymotrypsin[25–28] and trypsin[29,30] the uniquely reactive serine residue, which is phosphorylated when the enzymes are inhibited by DFP, is located in the sequence: Gly·Asp·Ser·Gly. It seems reasonable to suppose that this particular sequence of amino acids must play an important role in the esteratic action of these enzymes, and that the differences in their specificity must be due to some unique feature residing in the remainder of the molecule. Thus, it is tempting to speculate that the broadened specificity of ATd is the consequence of the removal of that portion of the molecule which restricts the action of the original enzyme to an ester bond derived from only lysine or arginine. The validity and general applicability of such a theory must await not only more extensive studies with ATd but also further work with other active degraded esterases in relation to substrate specificity.

It was hoped that the molecular weight of ATd could be estimated from the amount of *p*-nitrophenol instantaneously released from NPA, a technique which HARTLEY AND KILBY[31] had previously shown resulted in the rapid initial release of 1 mole of nitrophenol per mole of chymotrypsin. Although a nearly stoichiometric release of nitrophenol was obtained with trypsin and acetyltrypsin, ATd only enhanced the catalysis of NPA without causing a sudden, initial release of nitrophenol. The catalytic effect of ATd, however, was eliminated by treatment with DFP suggesting that the site of action of DFP on ATd is closely associated, if not identical, with the site involved in the hydrolysis of NPA. These and related observations have been studied in more detail and will be reported in a future communication.

In view of the implied association of histidine with the catalytic site of trypsin and other esterases[32-34], special efforts were made to establish with certainty the presence or absence of this amino acid in ATd. Quantitative chromatographic and microbiological assays of different preparations of ATd gave values ranging from 0.15 to 0.46 moles of histidine/6000 g protein. The fact that the expected 3 moles of histidine were recovered from acid hydrolysates of trypsinogen or the unfractionated peptic digest of acetyl trypsinogen would seem to rule out the possibility of significant destruction of histidine during hydrolysis of the protein. Since there appears to be no correlation between the histidine content and the specific activity of various preparations of ATd, it would seem that this amino acid does not participate in the catalytic function of this enzyme. The detection of non-stoichiometric quantities of histidine may simply be a reflection of peptide contamination which has been referred to earlier. At any rate, the precise significance of these results remains in doubt and continues to be the object of further investigation.

ACKNOWLEDGEMENT

This work was supported by grants from the National Institutes of Health (RG4614) and the National Science Foundation (G 5830).

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PURINE NUCLEOTIDE EXCRETION BY *ESCHERICHIA COLI* IN THE PRESENCE OF STREPTOMYCIN

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(Received April 25th, 1959)

SUMMARY

In the presence of a bactericidal concentration of streptomycin *E. coli* cells excrete considerable amounts of material with an absorption maximum at 260 mμ. The material has been separated chromatographically into two fractions. These have been identified by chromatographic and spectral properties, enzymic hydroysis, and analyses for pentose and phosphate, as 5′-adenylic acid and 5′-guanylic acid.

During the first hour of treatment there is no extensive breakdown or synthesis of nucleic acid in the cells. The excreted nucleotides are formed *de novo*, at a rate that approximates their normal rate of synthesis by growing cells.

INTRODUCTION

Although a number of effects of streptomycin on bacterial metabolism have been observed, none of these have explained the bactericidal action of this drug. Several considerations have suggested that it might be worthwhile to attack this problem by studying the effect of streptomycin on nucleic acid metabolism. Thus an interaction of streptomycin with nucleic acid in the cell has long been suspected on the basis of the polybasic structure of the drug, its ability to precipitate nucleic acid *in vitro*[1], and the marked susceptibility of its antibacterial action to interference by hydrogen ions and other cations[2,3] Furthermore, streptomycin abolishes inducible

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Abbreviations: RNA, ribose nucleic acid.

enzyme formation[4,5], which is ordinarily closely linked to ribonucleic acid synthesis.

This paper presents observations on the action of streptomycin on the nucleic acid metabolism of *Escherichia coli*. The initial observations were based on measurements of the absorption of a culture at 260 mμ compared with longer wave lengths. This simple technique has been shown to be quite sensitive to alterations in the nucleic acid concentration of bacteria[6]. No striking effect of streptomycin on the composition of the cells was observed, but considerable u.v.-absorbing material was found to accumulate in the culture medium. This material has been found to consist predominantly of two purine ribonucleotides, and some observations have been made on its origin.

MATERIALS AND METHODS

The experiments were performed with *E. coli*, strain W, in the minimal medium of DAVIS AND MINGIOLI[7] containing 0.5 % glucose. Cultures were incubated at 37° in aerobic shaken flasks.

O.D. was measured at $\lambda = 490$ mμ with a Lumetron colorimeter model 401A. Viable organisms were estimated by diluting in distilled water or phosphate buffer and plating on tryptic digest agar. Colonies were counted after incubation of the plates for 18 h at 37°.

The streptomycin used was Squibb streptomycin sulfate.

Inorganic and organic phosphate were determined by the method of FISKE AND SUBBAROW[8]. Pentose was estimated by a modification of the procedure of MEJBAUM[9] using D-ribose as standard. Deoxyribose was estimated by the BURTON modification[10] of the DISCHE test, reagents and sample being allowed to react for 4 h at 37°. Deoxyribose served as standard.

For paper chromatography Whatman No. 3 paper was used. The three solvent mixtures employed were: (a) *n*-butanol–water–acetic acid (1 :1 :0.24); (b) isopropanol–HCl (170 ml isopropanol–41 ml conc. HCl–39 ml distilled water); (c) isobutyric acid–0.5 *N* ammonium hydroxide (10:6). For the most part ascending chromatography was used, usually for 12–18 h at room temperature, followed by drying at room temperature. To locate u.v.-absorbing materials the chromatograms were inspected under a broad spectrum u.v. lamp.

Authentic samples of adenine, guanine, cytosine and uracil were obtained from Nutritional Biochemical Corporation. The California Corporation for Biochemical Research was the source of 2′,3′-guanylic, adenylic, uridylic and cytidylic acids, as well as of adenosine-5′-phosphate and guanosine-5′-phosphate. On paper chromatography the samples each gave a single major spot whose R_F was in good agreement with published values.

[8-^{14}C]Guanine was obtained from the New England Nuclear Corporation.

Rattlesnake venom (*Crotalus adamanteus*) with phosphomonoesterase activity was obtained from the Ross Allen Reptile Institute, Silver Springs, Fla. To measure the action of this enzyme on various nucleotides 2 mg of venom was mixed with 1.0 mg of substrate in 1.0 ml of 0.1 *M* tris(hydroxymethyl)-aminomethane (pH 9) containing 0.009 *M* $MgSO_4$. After incubation at 37° for 15 min the reaction was stopped with 1.0 ml of 2 *N* acetic acid and the percent dephosphorylation was estimated from the inorganic phosphate liberated.

RESULTS

Accumulation of u.v.-absorbing material by streptomycin-treated E. coli

To study the metabolic effects of streptomycin, a concentration was chosen (60 μg/ml) which was rapidly bactericidal in the medium used. As is shown in Fig. 1, 30 min after this addition of the drug to an exponentially growing culture less than 10^{-3} of the cells remained viable.

As a first approach to studying the effect of streptomycin on protein and nucleic acid synthesis, observations were made on the O.D. of cultures at 260, 280, and 490 mμ. Typical results are presented in Fig. 2. In this experiment cells from an overnight culture of *E. coli* in minimal medium were inoculated into two flasks of the same medium. When growth was exponential and the cell density approached $5 \cdot 10^7$ cells/ml, streptomycin was added to one flask. Samples were withdrawn at intervals during further incubation, and absorption measurements were made both before and after removal of the cells by centrifugation. Whereas in the culture without streptomycin growth (measured by turbidity at 490 mμ) continued and the u.v. absorbance of the culture fluid showed little change in successive samples, in the treated culture there was only slightly further growth but the 260 absorbance increased sharply. The material responsible for this increased absorption was found to be present largely in the filtrate. Furthermore, the 260/280 absorbance ratio of the excreted material was high, suggesting preferential accumulation of nucleic acid or

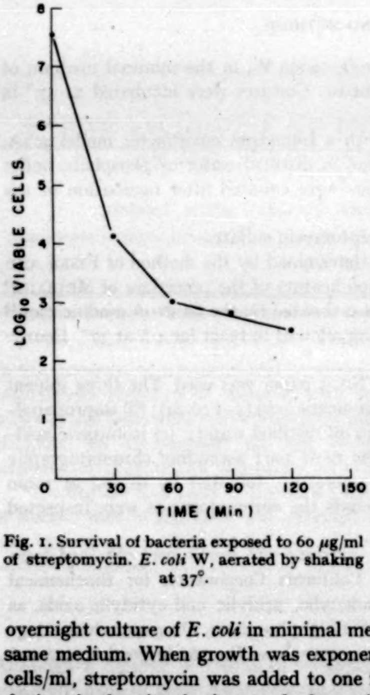

Fig. 1. Survival of bacteria exposed to 60 μg/ml of streptomycin. *E. coli* W, aerated by shaking at 37°.

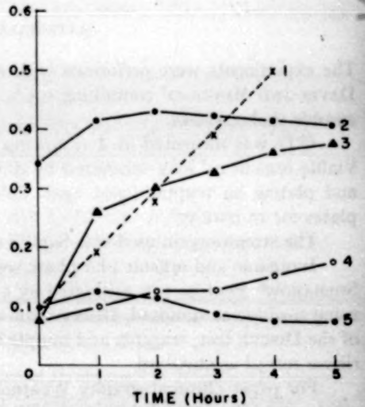

Fig. 2. Comparison of 260-absorbing material accumulated by streptomycin-treated and untreated cells. *E. coli* W, aerated by shaking at 37°. Curve: 1. Turbidity of untreated culture ($\lambda = 490$ mμ); 2. 260-absorption of resuspended cells from streptomycin-treated culture; 3. 260-absorption of filtrate from streptomycin-treated culture; 4. 260-absorption of filtrate from untreated culture; 5. Turbidity of streptomycin-treated culture ($\lambda = 490$ mμ).

its derivatives. The quantity of material excreted in 1 h corresponded, in its absorption at 260 mμ, to approximately half the nucleic acid content of the bacteria of the culture.

The u.v. absorption spectrum of the filtrate exhibited an absorption maximum at 260 mμ, but the curve did not approximate closely that of any known single purine or pyrimidine base or derivative[11].

A streptomycin-resistant mutant, exposed to streptomycin under the same conditions, was not killed and did not accumulate 260-absorbing material.

For further study of the excreted material streptomycin was added to 400 ml of a growing turbid culture of the wild type and 1 h later the culture filtrate was obtained. This material, and a parallel sample obtained from an untreated control culture, were each concentrated 100-fold under reduced pressure at 20°.

Identification of the excreted compounds

Addition of 5 % trichloracetic acid in the cold failed to produce turbidity with the concentrated filtrate from either the treated or the untreated culture. This procedure is sensitive to a protein concentration of 0.01 %[12]. It would also have revealed any substantial amounts of ribonucleic acid or deoxyribonucleic acid.

When the concentrated filtrate from the treated culture was chromatographed in butanol–acetic acid a single opaque spot ($R_F = 0.08$) could be observed under u.v. light. Chromatography in isopropanol–HCl, however, revealed two principal u.v.-absorbing spots, with the R_F values of adenylic acid (0.49) and guanylic acid (0.43), respectively. The material eluted from the two spots together represented about 90 % of the absorption of the filtrate at 260 mμ.

For purification and more definitive characterization of the compounds, an accumulation was carried out on a larger scale. Streptomycin (60 μg/ml) was added to an 8-l aerobic growing culture of *E. coli* and 90 min later the culture filtrate was obtained by centrifugation. Charcoal ("Norit A", 1 g/l) was added and after 45 min at room temperature, with agitation every 5 min, the charcoal was allowed to settle and the supernatant fluid was removed. The Norit was washed with 1 l of water and then extracted by shaking with four successive 100-ml portions of an aqueous solution containing 50 % ethanol and 5 % ammonium hydroxide. The combined extraction fluids were evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 5.0 ml of water, filtered to remove charcoal dust, and chilled overnight.

The total absorption of this material at 260 mμ was 80 % of that of the culture filtrate. The u.v.-absorbing components were adsorbed on a column of Dowex-2-chloride which had been washed free of excess acid. The column was then washed with distilled water and the nucleotide fraction was eluted with 60 ml of 0.1 *N* HCl. The eluate was evaporated to dryness *in vacuo* over anhydrous calcium sulfate and potassium hydroxide at 4°. The residue was redissolved in 8 ml of distilled water and this solution was used as starting material in the procedure described below.

Separation and identification of compounds by column chromatography

5 ml of the solution (absorption equivalent to approximately 10 mg of nucleotides was adsorbed on a column of Dowex-2-chloride and fractionally eluted with 0.003 *N* HCl. Two major fractions were obtained. The first to appear (Compound I) had u.v. absorption spectra at pH 2 and pH 7 which closely resembled those of adenylic acid.

The closeness of fit is indicated by the 250/260 and the 280/260 mμ absorbance ratios (pH 2) of 0.86 and 0.32, respectively, compared with 0.85 and 0.25 for authentic adenylic acid. Similarly, Compound II yielded spectra at the same pH values very similar to those of guanylic acid, with 250/260 and 280/260 absorbance ratios (pH 2) of 1.00 and 0.68 compared with 0.94 and 0.73 for authentic guanylic acid.

Using the known molar extinction value of adenylic acid ($\varepsilon_{260} = 14.2 \cdot 10^3$ at pH 2), it could be computed that 5.3 mg of Compound I was recovered from the Dowex column. A similar calculation, employing the value of guanylic acid ($\varepsilon_{260} = 11.8 \cdot 10^3$), indicated a recovery of 3.7 mg of Compound II.

Base, pentose, and phosphorus content of Compounds I and II

The concentrations of base in the solutions of Compounds I and II, calculated as just described, were compared with the concentrations of pentose residues and organic phosphate, determined as described under METHODS. The results yielded a molar ratio of base: pentose:phosphate of 1.0:0.9:1.2 for Compound I, and 1.0:0.95:0.85 for Compound II. No deoxyribose could be detected in either compound.

Position of phosphate of nucleotides

The results described support the formulation of Compound I as adenylic acid and Compound II as guanylic acid; but they do not discriminate between the 2′, 3′, and 5′ forms of the nucleotides. MOYED AND MAGASANIK[13] have shown that 5′-purine nucleotides move differently from their 2′ or 3′ isomers in ascending chromatography in a solvent system of isobutyric acid–ammonia. In this system Compounds I and II were found to move like 5′-adenylic and 5′-guanylic acids, respectively*. In addition, a mixture of the accumulated compound with the appropriate 5′-nucleotide gave a single spot, while a mixture with the 2′, 3′-nucleotide gave a double spot. Finally, decisive evidence for the 5′-nucleotides was obtained by treatment with rattlesnake venom phosphomonoesterase[14]. Compound I was hydrolyzed to about the same extent as a similar quantity of adenosine-5′-nucleotide (43 % compared with 61 %), while a mixture of 2′ and 3′ adenine nucleotides was not detectably hydrolyzed. Similarly, Compound II and authentic guanosine-5′-phosphate were 63 % and 68 % hydrolyzed, while a guanine-2′,3′-nucleotide mixture showed no hydrolysis.

Thus, in Compound I and Compound II the known components and certain distinctive properties of adenosine-5′-phosphate and guanosine-5′-phosphate, respectively, have been demonstrated; and the R_F values are considered to exclude either polymerization of the mononucleotides or the presence of additional, unrecognized residues. It is concluded that the excreted compounds are adenosine-5′-phosphate and guanosine-5′-phosphate.

Origin of the excreted nucleotide

Since the streptomycin-induced excretion of nucleotides was not accompanied by significant loss of the absorption of the cells at 260 mμ, or by excretion of comparable quantities of pyrimidines, it seemed unlikely that the observed excretion was based on breakdown of cellular nucleic acid. In order to obtain decisive evidence on this point, experiments were carried out with an isotopically labelled purine. A culture was grown from a small inoculum in the presence of [8-^{14}C]guanine (20 μg/ml; 15,000 counts/min/μmole). When the culture attained a population density of $6 \cdot 10^7$ bacteria/ml the cells were centrifuged and washed twice, each time in one-half the original volume of water. Half the cells were saved and half were inoculated into fresh minimal medium ($6 \cdot 10^7$ cells/ml) with streptomycin and returned to the shaker at 37°. After 90 min of incubation the bacteria and the filtrate were harvested and the cells were washed with water.

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* The R_F values observed for Compound I, adenosine-5′-phosphate, and an adenosine-2′, 3′-phosphate mixture were, respectively, 0.43, 0.43, and 0.50. For Compound II and the guanosine 5′- and 2′,3′-nucleotides the values observed were, respectively, 0.26, 0.26, and 0.30.

To determine the specific radioactivity of cellular guanine the streptomycin-treated and the untreated cells were hydrolyzed in 5.0 ml of 5 % trichloracetic acid at 100° for 30 min. After cooling and then centrifugation at 5000 × *g*, the supernatant fluid was recovered and the trichloracetic acid was partly removed by several extractions with ether. The aqueous phase was reduced in volume to about 1.0 ml by evaporation at 45° with a stream of air. Of this solution 0.05 ml was applied to paper and chromatographed in isopropanol–HCl. The guanine spot was eluted from the paper in 1.5 ml of 0.01 *N* HCl; its u.v. extinction corresponded to 20 to 40 μg of guanine. The material was then evaporated to dryness on a stainless steel planchet and the radioactivity was estimated in a windowless gas flow counter. The specific activity of the cellular guanine was calculated from these data (Table I).

TABLE I

ORIGIN OF EXCRETED GUANINE

Experiment No.	*Specific activity (counts/min/µmole guanine)*			
	Guanine in growth medium	*Cell guanine before SM*	*Cell guanine after SM*	*Guanine excreted as nucleotide*
1*	15,000	5,800	6,000	400
2**	15,000	5,400	4,800	1,100

* Experiment 1: cells exposed to streptomycin for 90 min before harvesting filtrate.
** Experiment 2: cells exposed to streptomycin for 120 min before harvesting filtrate.

In the untreated cells approximately 39 % of the guanine appears to have been supplied by the exogenous labelled guanine in competition with that derived from unlabelled glucose. After exposure to streptomycin the specific activity of cellular guanine was essentially unchanged. It follows that during this treatment there was little dilution of pre-existing labelled cellular guanine, either by turnover or by net synthesis of nucleic acid.

To assess the specific activity of the excreted guanylic acid, the filtrate of the treated culture was mixed with Norit, as described above for the isolation of the accumulated compounds. The nucleotide fraction was eluted and then hydrolyzed in 5 % trichloracetic acid to liberate the bases. The guanine was separated chromatographically and its specific radioactivity was determined in the same manner as described for cellular guanine. The activity of the excreted guanine in experiment 1 of Table I was approximately 7 % of that of cellular guanine. In experiment 2, which was identical except for a longer period of exposure to streptomycin, a larger portion of the excreted material was derived from cellular guanine. It is concluded that the guanine released from the cells during early incubation with streptomycin does not arise to a significant extent from the breakdown of preformed nucleic acid*.

In some experiments uridylic acid and uracil were found in the culture filtrates after streptomycin treatment. These did not, however, always occur in detectable quantity, and they never exceeded 10 % of the purine nucleotides in fluids harvested up to 3 h after addition of the drug.

To study further the time relations of purine and pyrimidine excretion, hourly samples were taken from a large culture incubated for 6 h with streptomycin as before. The supernatant fluids were concentrated and analyzed by paper chromatography and elution.

Two such experiments showed that the purine nucleotides appeared early but the pyrimidines began to appear only as the bacteria started to lyse (as indicated by a drop in turbidity of the culture at 490 mμ). The excretion of pyrimidines increased as lysis progressed, and was accompanied by increasing amounts of the 2′,3′ mixtures of adenylic and guanylic acids (paper chromatography in isobutyric acid–ammonia).

DISCUSSION

The present work has shown that in the presence of streptomycin, in a concentration that prevents growth and causes rapid loss of viability, *E. coli* cells excrete into the medium a mixture of 5′-adenylic and 5′-guanylic acids. These compounds have been identified by their u.v. absorption spectra, their chromatographic mobility in various solvents, their base : pentose : phosphate ratios, and their hydrolysis by snake venom phosphomonoesterase.

It seemed likely that the excreted purine nucleotides arose by *de novo* synthesis rather than from breakdown of preexisting RNA, since the u.v. absorption of the cells ($\lambda = 260$) did not decrease as that of the medium increased, and since the excreted purine nucleotides were not accompanied by pyrimidines (in nucleotide or other form). This conclusion was confirmed by the results of experiments with [^{14}C]guanine. In 90 min of incubation with streptomycin less than 10 % of the excreted guanine arose from guanine previously incorporated in the cell; and part of this small fraction undoubtedly arose from the intracellular nucleotide pool. The fact that the excreted compounds are 5′-nucleotides is also consistent with their *de novo* origin; breakdown of ribonucleic acid by the usual ribonucleases would be expected to yield 2′ and 3′-nucleotides (as did, in fact, occur on more prolonged incubation with streptomycin).

In prolonged experiments (*e.g.*, more than 2 h) moderate excretion of uracil and uridylic acid was also observed. Furthermore, the contribution of cellular guanine to the excreted material became larger in a more prolonged experiment. Compared with the early excretion of purine nucleotides, these delayed phenomena are undoubtedly remote in origin from the immediate action of streptomycin; they probably reflect, like the decreasing turbidity of the culture, slow lysis several hours after the lethal action of streptomycin.

The excretion of these purine-5′-nucleotides was substantial. In 1 h it approximately equalled the total purine content of the cells at the time of addition of streptomycin. Slower further excretion continued for several more hours.

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* **In an experiment identical with experiment 2 of Table II the filtrate was examined chromatographically and was found to contain a significant quantity of guanosine-2′,3′-phosphate. It therefore seems probable that the isotopic data of experiment 2 reflect some breakdown of ribonucleic acid late in the experiment.**

The continued synthesis of these major cell constituents for 1 h, at approximately the same rate as in a growing cell, would appear to exclude any mode of action of streptomycin that would severely impair the supply of energy for biosynthesis.

With lethal agents it is difficult to determine whether a given alteration in the cell is a primary or a more remote consequence of the action of the compound. In the case of streptomycin the excretion itself is not necessary for the lethal action. Thus when a purine auxotroph of *E. coli* was exposed to the drug in minimal medium, in which its growth was prevented by the absence of purine, there was no significant excretion of purines; yet the bactericidal action was as rapid as that observed with the wild type (ROTH AND DAVIS, unpublished observation). Nevertheless, it seems possible that the excretion of purine nucleotides (observed when they can be formed) reflects the biochemical lesion closely enough to be useful in pursuing the action of streptomycin. An analogy is the accumulation of characteristic compounds in the presence of penicillin[15]. Whether the excretion of purine nucleotides is a consequence of an alteration in their formation, utilization, or retention, is under investigation.

NOTE ADDED IN PROOF

In the present work the excretion of purine nucleotides appeared to be specific, since pyrimidine nucleotides were not consistently observed before 2 h or more of streptomycin treatment. However, subsequent work (N. ANAND and B. D. DAVIS, unpublished observations), employing column chromatography to separate the nucleotides, has shown that pyrimidine nucleotides are also excreted early, though in smaller quantities than purine nucleotides. It is thus doubtful that the streptomycin effect distinguishes sharply between these compounds.

(Received November 19th, 1959)

ACKNOWLEDGEMENTS

This work was aided by U.S. Public Health Service Grants E-2012 and E-957 C2 and the Eugene Higgins Trust. H. R. is a Post-Sophomore Fellow, U.S. Public Health Service (New York University College of Medicine). H. A. is a Senior Research Fellow (SF-124) of the U.S. Public Health Service.

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DNA SYNTHESIS IN INDIVIDUAL L-STRAIN MOUSE CELLS

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(Received April 21st, 1959)

SUMMARY

The time relationship between DNA synthesis and mitosis has been determined for L-strain mouse cells cultivated *in vitro*. DNA synthesis was detected autoradiographically by following the uptake of [^{3}H]thymidine into the cell nucleus. DNA synthesis in this cell system was found to take place in an approximately linear fashion over a single period of six to seven hours, ending three to four hours before mitosis, for a total generation time of twenty hours.

A mathematical treatment for exponentially multiplying cultures is presented.

INTRODUCTION

It is obvious that a cell must double its content of deoxyribose nucleic acid (DNA) before it divides. For many years it was believed that the cell synthesized this DNA during prophase and metaphase, so that when the two sets of chromosomes separated at anaphase, each had a full complement of DNA. Recently, many workers have shown, by the use of photometric and autoradiographic techniques, that DNA synthesis actually occurs during interphase over a period which ends at a certain time before mitosis, and which has a definite length[1–7]. This was found to be true for many different types of cells, both animal and plant, but the exact timing of the DNA synthesis period appeared to vary with the cell type. We have investigated, by the method of autoradiography, DNA synthesis in exponentially multiplying mouse cells, cultivated *in vitro*. This investigation is preliminary to studies of the perturbing influence of various chemical and physical agents upon DNA metabolism in the cell.

MATERIALS AND METHODS

Detection of DNA synthesis

Since thymidine has been shown to be a specific precursor of DNA[8], the uptake of [^{3}H]thymidine (^{3}HTDN) into the cell nucleus was used as a measure of DNA synthesis. The β-decay of tritium was detected autoradiographically. The low energy of tritium β-particles affords excellent resolution on the autoradiographs[9] (see Fig. 1).

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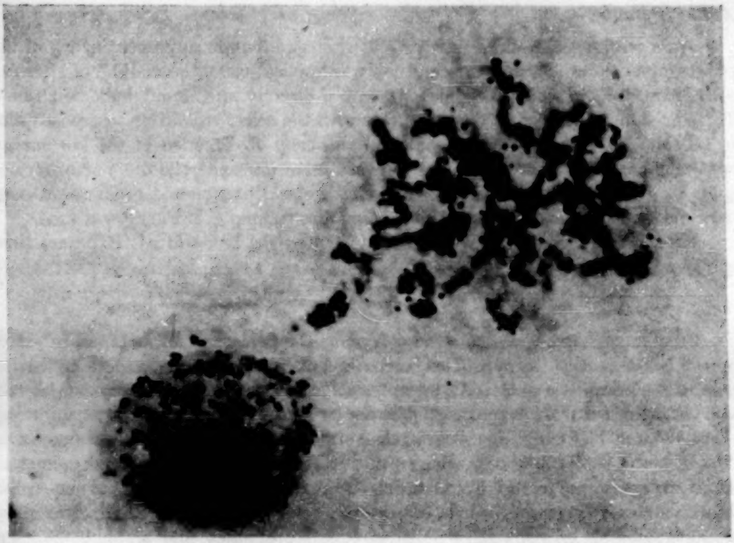

Fig. 1. Autoradiograph of two L cells after 16-h exposure to [^{3}H]thymidine. One cell is in metaphase, the other in interphase. The metaphase cell has synthesized one complement of DNA in the presence of label. Film exposure time was 15 days.

Cells

EARLE's L-strain mouse cells (NCTC Clone 929) were used[10]. Cells were cultivated continuously in the logarithmic growth phase, using suspension cultures[11]. It has been shown that under these conditions, almost 100% of the cells are viable[12]. Growth medium for stock cultures consisted of a 20% solution of horse serum in CMRL 1066[13]. For labelling of cellular DNA, ^{3}HTDN at a specific activity of 360 mC/mmole* was added to the growth medium to a concentration of 0.25 μC/ml. In one experiment a concentration of 0.5 μC/ml was used. The effective specific activity of the ^{3}HTDN was reduced 40-fold due to the presence of 10 μg/ml of unlabelled thymidine in the growth medium. The concentration of ^{3}HTDN used was safely below the radiation toxic level reported by PAINTER, DREW AND HUGHES[14] for HeLa cells, and no effect of addition of ^{3}HTDN on the growth rate of the cultures was observed.

Preparation of slides for autoradiography

After addition of ^{3}HTDN, the culture was sampled at hourly intervals. The samples taken were small, so that a single suspension culture could be used for an entire experiment. The cells of each sample were immediately dilated in hypotonic saline and fixed in acetic–alcohol, whereupon aliquots were smeared on duplicate slides and allowed to dry. The slides were then washed in acetic–alcohol, stained with 2% acetic orcein, washed again in alcohol to remove excess stain, and then washed in running water for 3 to 4 h to prepare them for autoradiography.

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Autoradiography

After washing, the slides were ringed with egg albumin to prevent lifting of the photographic emulsion, and the emulsion was applied, using the method of MESSIER AND LEBLOND[15], in which the slides are dipped directly into liquid bulk emulsion*. The emulsion was placed in direct contact with the cells, without an interposed film of celloidin, in order to ensure maximum efficiency of detection of the low energy tritium β-particles[16]. Direct contact between emulsion and cells did not introduce spurious grains, as unlabelled cells showed a normal background grain count over their nuclei. In one experiment, the stripping film technique of PELC[17] was used.

For exposure, the slides were stored in light tight boxes at 4°. Exposure time was usually two weeks.

Counting methods

Fifty metaphases were scored to determine the percentage of metaphases which were labelled in each sample. The percentage of labelled cells was determined by scoring 500 interphase nuclei. An interphase nucleus or a metaphase was considered to be labelled if it was covered with 5 or more grains, the average background being about 2 grains/nucleus. The generation time of the cells was determined by measuring the cell concentration in the growing culture at various times with a hemocytometer. Mitotic indices were measured by counting the number of cells in mitotis in 3000 cells. In our material, the great majority of cells in mitosis were found to be in metaphase; one slide showed, for example, 73 metaphases, 7 prophases, and 2 anaphases. Our mitotic indices are therefore, to a close approximation, the percentage of cells seen in metaphase. Any cell showing a single group of clearly discernible chromosomes in the absence of a nuclear membrane was considered to be in metaphase.

RESULTS

From the recent reports of interphase DNA synthesis in various types of cells referred to in the introduction, it was assumed at the outset that every cell in the exponentially growing culture passes repetitiously through a mitotic cycle of the type illustrated in Fig. 2. If cells are considered to move clockwise around the cycle, they pass successively through a postmitotic non-synthetic period (G_1), a period of active synthesis of DNA (S), and a premitotic non-synthetic period (G_2)[1,3,4,7]. It remained to verify this pattern of DNA synthesis in our cells and to measure the actual values of G_2, S, and G_1. We also investigated how DNA synthesis proceeds with time over the S period.

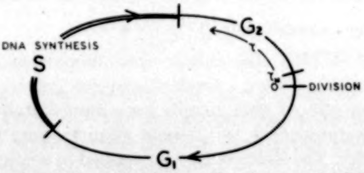

Fig. 2. The mitotic cycle of actively multiplying cells, showing the DNA synthetic period and its time relation to division (cells move clockwise around the cycle).

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* Ilford Nuclear Research Emulsion, type G5, in gel form, Ilford, Inc., Ilford, Essex.

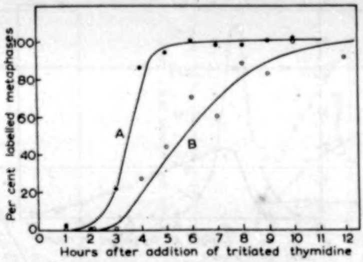


Fig. 3. The percentage of labelled metaphases for two cultures (denoted A and B) at various times after addition of [^{3}H]thymidine.

Determination of the G_2 *period*

The time required for labelled DNA to show up in metaphase chromosomes should give a measure of the length of the G_2 period. The ratio of labelled metaphases to total metaphases, labelled or unlabelled ("percent labelled metaphases") was measured for each hour after addition of ^{3}HTDN. Fig. 3 gives the results of two experiments. It is to be seen that in both cases virtually no labelled metaphases are seen for 3 h after addition of label. That this is not a lag in the uptake of label is shown by the observation that many *interphase* nuclei have taken up label within 1 h after addition of ^{3}HTDN (see Figs. 8 and 10). Indeed, the lag in uptake of label is probably negligible[7]. The 3-h delay in the appearance of label in cells in metaphase means that the period between completion of DNA synthesis and metaphase, *i.e.*, the G_2 period (see Fig. 2), is at least 3 h long. This, however, is a minimum period only. If all the cells had the same 3-h G_2 period, all metaphases would be labelled, though only slightly so, when this period had elapsed; that is, all metaphases seen at time G_2 after the addition of label would have just been completing DNA synthesis when ^{3}HTDN was added, and would thereby all be labelled. The percent labelled metaphases curve would, after a 3-h interval, rise abruptly to 100 %. The fact that it does not indicates that there is a variation among cells in the length of the G_2 period. Since, as will be shown, the duration of metaphase is short, about 0.5 h, the variation seen in Fig. 3 is only in a small part due to the time required for cells to pass through metaphase. A larger variation was observed in experiment B than in experiment A.

By taking the derivative of the percent labelled metaphase curves, the frequency distributions relating the numbers of cells with a given G_2 period are obtained. These are presented in Fig. 4. In experiment A the result is a sharp Gaussian curve with a peak at 3.5 h; in experiment B it is a more flattened curve skewed to the right, with a peak near 4 h. The reason for the difference between the two experiments has not been ascertained, but is probably due to differing culture conditions.

The generation time was found to be 20 h for both experiments. Thus, for a generation time of 20 h, the G_2 period has a most probable value of from 3 to 4 h.

Determination of the S *period*

The length of the G_2 period has now been found. The length of the S period can be determined in two ways.

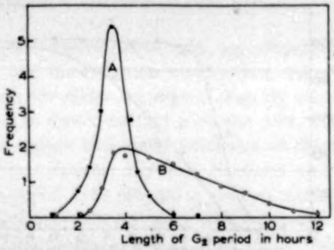


Fig. 4. Frequency distributions giving the relative number of cells with a given G_2 period for cultures A and B.

Grain counts over metaphases: The grain count over metaphases seen at increasing times after addition of label, should be zero (neglecting background) until the G_2 period has elapsed, then should increase for a time equal to the *S* period, after which it should increase no further since all the cells in metaphase after this time would have synthesized a full complement of DNA in the presence of label. The time between the first increase in the grain count over background and the time when the grain count saturates gives the length of the *S* period, and the intermediate grain counts give the manner in which DNA synthesis proceeds with time over the *S* period. Unfortunately, all the metaphases do not have the same number of grains over them at any time after addition of label, because there is variation in the length of the G_2 period, there is autoradiographic variation, and there is possible variation in DNA synthesis rates amongst cells. If, however, a distribution of the number of metaphases which have a grain count within a given interval is plotted for each time, the peak grain count values of these distributions should show the time dependence which would have been observed had there been no variation in the metaphase grain counts.

The slides of experiment A, which showed the smaller variation in the G_2 period, were selected for this experiment. Duplicate slides were used, one set being exposed for 15 days, the other for 8 days. Grains were counted over 50 metaphases generally, for each hour after addition of ^{3}HTDN, and a histogram of the number of metaphases within a given grain count interval plotted. Fig. 5 shows two such histograms for

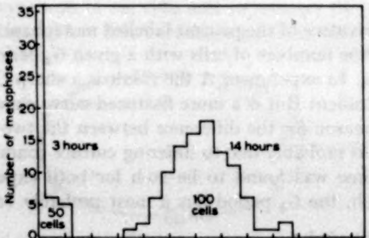
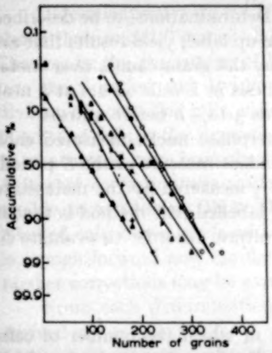


Fig. 5. Grain counts over individual metaphases; histograms of the number of metaphases within a given grain count interval for 3 h and for 14 h after addition of [^{3}H]thymidine.



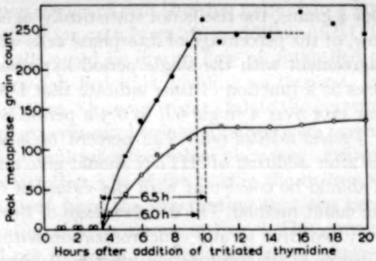


Fig. 7. Peak metaphase grain count values for various times after addition of [^{3}H]thymidine. Film exposure time for the upper curve was 15 days, lower curve, 8 days.

Fig. 6. The percentage of metaphases with a grain count up to and including the abscissa grain count values, plotted on probability paper. A Gaussian distribution appears as a straight line with its peak value at the 50% intercept. Six lines are given, corresponding to six times after addition of [^{3}H]thymidine: ▲, 5 h after addition of ^{3}HTDN; +, 6 h; ×, 7 h; △, 8 h; ●, 9 h; ○, 14 h.

3 and 14 h after addition of label. The 14-h distribution is a Gaussian, as were the distributions for all other times where all the metaphases were labelled.

The experimentally determined grain count distributions for each successive hourly sample were plotted on probability paper. Several of these are depicted in Fig. 6. When plotted on probability paper, a Gaussian distribution appears as a straight line, the peak value being given by the 50 % intercept of the line. The peak grain counts determined in this way for each hour after addition of ^{3}HTDN is plotted in Fig. 7. This method of determination of the peak grain count was not applied to the distributions for hours 1 to 4, since a Gaussian distribution would not be expected until all metaphases were labelled.

In Fig. 7, the upper curve represents peak grain counts as a function of time for a 15-day exposure, and the lower curve for an 8-day exposure of duplicate slides from the same experiment (A). Since grains are most conveniently counted when they number about 100 over a metaphase, the upper curve is more reliable for $t < 8$ h and the lower curve is more reliable for $t > 8$ h. It is seen that the metaphase grain count saturates in both cases 9.5 to 10 h after addition of ^{3}HTDN. The DNA synthetic period is therefore 6 to 6.5 h long.

From Fig. 7, it is seen that DNA synthesis is close to being linear with time over the *S* period. The curves are slightly concave which may indicate the DNA synthesis occurs at a slightly greater rate near the end of the *S* period than at the beginning, though the significance of the shapes of the curves is doubtful. From the lower curve, which gives a more reliable saturation plateau, it is seen that the peak metaphase grain count is virtually constant from 10 to 20 h, which is the generation time. The slight rise occurring from 16 to 20 h, which represents an increase of about 6 % in the total grain count, could be due either to a second period of DNA synthesis at a very reduced rate, or to the presence of a few cells in the population which have short enough generation times to have entered the *S* period a second time. In point of fact, since the standard deviation of the peak grain count values for the lower curve is about 4 grains, the rise is not statistically significant. Determinations, to be described below, of the percentage of interphase cells which take up label, yield results that are in agreement with the single period hypothesis. Thus, the grain counts over metaphases as a function of time indicate that DNA synthesis in L-cells occurs at a near linear rate over a single 6.0 to 6.5-h period which ends 3 to 4 h before mitosis.

Percent labelled cells: The percent of labelled interphase nuclei measured each hour after addition of ^{3}HTDN should give an independent measure of the S period, and should be consistent with the values of G_1 and G_2 measured by the metaphase grain count method. The disadvantage of the percent labelled cells method is that it is very sensitive to any synchronization within the culture. In order to evaluate S, the degree of synchronization must be determined.

Asynchronous culture

We shall define an asynchronous culture as one in which the number of cells increases in a perfectly smooth exponential fashion with time, that is, one in which there is a constant fraction of cells undergoing division. For such a culture the frequency distribution of cells around the mitotic cycle (Fig. 2), or the number of cells in each portion of the cycle, is an exponential function[18] (see APPENDIX) with twice as many cells leaving mitosis as entering it, and the mitotic index is constant. It will be seen that a determination of the mitotic index as a function of time provides a sensitive indicator of the degree of synchronization present in a culture.

In experiment B, the mitotic index was found to be nearly constant with a value of about 1 % over a generation time of 20 h. It can thus be treated as an asynchronous culture. The length of time τ_m which the cells spend in mitosis for such a culture can be evaluated by applying eqn. (7) of the APPENDIX:

$$\tau_m = \frac{MT}{0.693}$$

where $M \times 100$ is the mitotic index and T is the generation time. This equation yields a value of τ_m of about 0.3 h. This is probably a minimum value for τ_m since slides that have not undergone preparation for autoradiography show mitotic indices of from 2 to 3 % *i.e.* a τ_m of about 0.7 h. However, since in this experiment the mitotic index obtained under constant conditions of slide preparation was found not to vary, it is valid to treat the culture as asynchronous.

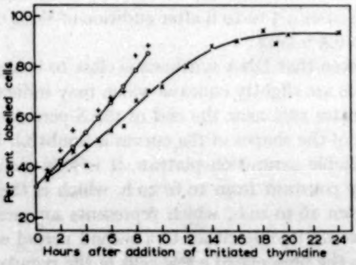


Fig. 8. The percentage of labelled cells for an asynchronous culture (B) at various times after addition of [^{3}H]thymidine. ×, experimental curve; O, the theoretical curve calculated from eqn. (14) using $S = 7.1$ h. For comparison, the corrected experimental points, +, are shown.

Determinations were made of the percentage of cells showing more than 5 grains above their nuclei ("percent labelled cells") for each hour after addition of ^{3}HTDN. Changes in the percentage of labelled cells could be due to flow of unlabelled cells into the S period and division of already labelled cells, yielding an increase, or to division of unlabelled cells, yielding a decrease. Since we wish to relate the percentage of labelled cells to the S period only, the experimental values obtained were corrected to what would have been observed had no multiplication taken place (see APPENDIX). A further correction was made for the fact that 5% of the cells in the culture were unable to synthesize DNA. This is evident from the observation that the percent labelled cells curve levels off at 95% after 16 h, as shown in Fig. 8. This correction is straightforward and the details will not be given. As discussed in the APPENDIX, further corrections may be avoided by using only those values for times less than G_1.

From each determination, a value of S was calculated by applying eqn. (15) derived in the APPENDIX:

$$S = \frac{1}{\alpha} \ln \left[L(t)_{\mathrm{corr}} + e^{\alpha G_2} \right] - (G_2 + t), \qquad t < G_1$$

where $\alpha = \ln 2/T$, T is the generation time, and $L(t)_{\mathrm{corr}} \times 100$ is the corrected percentage of labelled cells. ^{3}HTDN was added at $t = 0$.

The results are tabulated in Table I. The constancy of S serves as a test of the formulation.

TABLE I

EVALUATION OF THE S PERIOD

Time *h*	*Asynchronous culture (B)*		
	Experimental percentage labelled cells	*Corrected percentage labelled cells*	*Value of S (from eqn. (15))* *h*
1	36	39	7.2
2	42	47	7.6
3	47	55	7.9
4	41	49	6.0
5	51	63	7.3
6	55	68	7.1
7	57	71	6.5
8	67	85	7.6
			Mean: 7.1 h

From Table I, the average value found for S was 7.1 h. Using this average value for S in eqn. (14) of the APPENDIX the "theoretical" curve shown in Fig. 8 was constructed. Also shown are the corrected points and the uncorrected experimental curve.

Comparison of the curves shows that the results are consistent with the hypothesis that a single period of DNA synthesis exists for all cells, the period being about 7 h in duration and ending approx. 4 h before division.

Partially synchronized culture

If the mitotic index of a culture is not constant with time, the frequency distribution relating the number of cells within each portion of the cell cycle is not exponential. This can have a very pronounced effect upon quantities which depend upon the distribution, such as the percent labelled cells.

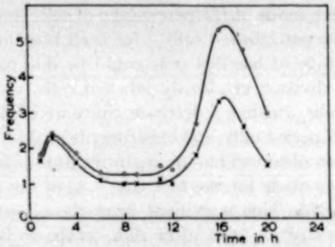


Fig. 9. Lower curve: the mitotic index at times after addition of [^{3}H]thymidine for a partially synchronized culture (A). Upper curve: the derived frequency distribution $n(t, 0)$, giving the relative number of cells in each portion of the mitotic cycle for the same culture.

The mitotic index of experiment A was found to vary with time as shown in Fig. 9. The slides of this experiment were used to determine the S period by the metaphase grain count method, though it should be emphasized that the mitotic variation would have no effect upon this determination, since the treatment is not dependent on the distribution of cell numbers in the various portions of the mitotic cycle. Nevertheless, as a check on the grain count method, an independent estimate of the duration of the S period may be obtained from a count of the percent labelled cells, and this does depend strongly on the distribution of cell numbers throughout the mitotic cycle. An analysis of this dependence is outlined in the APPENDIX.

Assuming that all the cells move around the mitotic cycle at the same rate, the relative number of cells in each portion of the cycle at $t = 0$ is given by $n(t,0)$, where t represents the time separating a particular cell from division. $n(t,0)$ is related to the measured mitotic index $M(t)$ according to eqn. (9) of the APPENDIX:

$$n(t,0) = \frac{1}{\tau_m} N_0 \, M(t) \exp \frac{1}{\tau_m} \int_0^t M(t)\mathrm{d}t$$

where N_0 is the total number of cells anywhere in the cycle at time $t = 0$, and

$$\tau_m = \frac{1}{\ln 2} \int_0^T M(t)\mathrm{d}t$$

where T is the generation time.

The percentage of labelled cells was corrected to what would have been observed in Fig. 9, yield a value of 0.5 h for τ_m. The values derived for $n(t,0)$ using eqn. (9) are also plotted in Fig. 9.

The percentage of labelled cells were corrected to what would have been observed had no cell multiplication taken place. The procedure for this correction is given in the APPENDIX. Since the experimental $L(t)$ curve reached virtually 100 % after about 15 h, no correction was required to allow for cells unable to synthesize DNA. The corrected percent labelled cell values, as a function of time, $L(t)_{\mathrm{corr}} \times 100$, can be predicted from $n(t,0)$ using eqn. (13) of the APPENDIX, by assuming a value for S:

$$L(t)_{\mathrm{corr}} = \frac{\int_{G_1}^{G_1+S+t} n(t,0)\mathrm{d}t}{N_0}, \qquad t < G_1$$

The value of S which gave best agreement between corrected experimental and

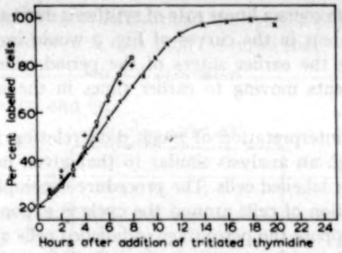


Fig. 10. The percentage of labelled cells for a partly synchronized culture (A) at various times after addition of [^{3}H]thymidine. ×, experimental curve; ○, the theoretical curve calculated from eqn. (13) using $S = 8.0$ h. For comparison, the corrected experimental points, +, are shown.

TABLE II

EVALUATION OF THE *S* PERIOD

Time h	*Partly synchronized culture (A)* Experimental percentage labelled cells	Corrected percentage labelled cells	Value of *S* (from eqn. (13)) (h)
1	26	27	8.3
2	33	36	8.8
3	34	38	8.2
4	44	51	8.4
5	49	55	7.8
6	58	65	7.8
7	65	73	7.6
8	71	80	7.4
			Mean: 8.0 h

theoretical $L(t)$ values, was determined for each hour, the integration being done graphically. These values are tabulated in Table II. The calculated values of $L(t)$ obtained using the mean value of S from Table II are plotted in Fig. 10, along with the corrected points and uncorrected experimental curve.

The mean value obtained for S was 8 h. This is somewhat larger than the value of 6.0 to 6.5 h obtained for metaphase grain counts performed on the same set of slides. This discrepancy may be due either to the assumptions inherent in the analysis, or to errors in the mitotic index values. It is quite difficult to obtain a reliable measure of the mitotic index, due to statistical errors and preparational inconsistencies.

DISCUSSION

The mitotic cycle found for L-strain mouse cells is similar to those reported by other workers for mammalian cells cultivated *in vitro*[4,7] in that they are characterized by a long post mitotic non-synthetic phase (G_1) involving approximately half of the total generation time, followed by a period of DNA synthesis (S) with a duration of about one-third of the generation time, followed in turn by a short premitotic non-synthetic period (G_2) which lasts for about one-fifth of the generation time. The division process itself is rapid, requiring less than an hour for completion.

The evidence also favours a linear rate of synthesis during the S period. The slight decrease in slope apparent in the curves of Fig. 7 would imply a decreased rate of DNA synthesis during the earlier stages of the period, since moving towards the right in Fig. 7 represents moving to earlier times in the mitotic cycle, *i.e.* away from mitosis.

The quantitative interpretation of much data relating to exponential cultures is only possible through an analysis similar to that given in the APPENDIX for the rate of accumulation of labelled cells. The procedure is complicated by the fact that the frequency distribution of cells around the cycle is exponential rather than constant. For example, suppose the percentage of labelled cells after a brief exposure to label for an asynchronous culture with a generation time of 20 h, was found to be 30 %. The length of the S period is not given by taking $0.30 \times 20 = 6$ h which assumes a constant distribution, but by eqn. (15), which yields $S = 6.7$ h, a difference of over 10 %. An exponential distribution, also, can be expected only when the mitotic index is constant with time. If it is not, the distribution must be evaluated (see APPENDIX), since cyclic quantities are very dependent upon it. For example, in one partially synchronized culture, the initial percent of labelled cells was found to be 20 %, which, using eqn. (15), gives $S = 4.7$ h. When the proper distribution was evaluated, S was calculated to be 7.3 h.

From the measured values of the rate, duration and location within the mitotic cycle of the S period, the average DNA content of a cell in relation to its premitotic and postmitotic DNA content can be calculated. This calculation is presented in the APPENDIX, and yields the result that the average content is 65 % of the premitotic content.

ACKNOWLEDGEMENT

This work was aided in part by a grant from the National Cancer Institute of Canada.

APPENDIX

Distribution of cell numbers at different portions of the mitotic cycle

Asynchronous case: In an asynchronous, exponentially multiplying culture, the cell concentration $N(t)$ as a function of time t is given by:

$$N(t) = N_0 e^{at} \tag{1}$$

Where N_0 is the initial concentration and $a = 0.693/T$, T being the doubling time.

To locate the position of any given cell within the mitotic cycle of Fig. 2, a variable τ, where $0 < \tau < T$ is assigned, representing the time separating the cell from division. At $\tau = G_2 + S$, the cell begins DNA synthesis which lasts a time S; at $\tau = G_2$ the cell ceases DNA synthesis and enters the premitotic non-synthetic period of duration G_2; at $\tau = \tau_m$, the cell enters mitosis and at $\tau = 0$ the cell divides. Thus, the G_2 period is defined as the period from completion of synthesis to division.

Let $n(t, \tau)$ be the number of cells per unit time at time t flowing through a point on the cycle which precedes division by a time τ. At $\tau = 0$, the number of cells dividing in a time interval t to $t + dt$ is $dN(t)$, the number of new cells appearing:

$$n(t, 0)\, dt = dN(t) \tag{2}$$

From eqn. (1),

$$n(t, 0) = aN_0 e^{at} = aN(t) \tag{3}$$

Assuming a uniform rate of flow of cells around the cycle, a cell which is at position τ at a time t will have moved to $\tau = 0$ at time $t + \tau$, so that:

$$n\,(t + \tau,\, 0) = n(t,\, \tau) \tag{4}$$

which yields, using eqns. (1) and (3),

$$n\,(t,\, \tau) = \alpha N(t)\, \mathrm{e}^{\alpha \tau} \tag{5}$$

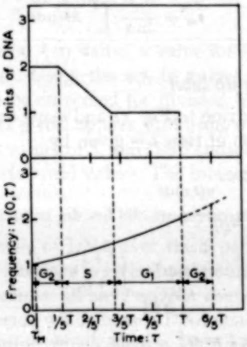


Fig. 11. Lower graph: the frequency distribution of an asynchronous culture giving the relative number of cells in each part of the mitotic cycle and showing the approximate position of the G_2, S and G_1 periods. Upper graph: The DNA content, in arbitrary units, of cells in the G_2, S and G_1 periods.

In Fig. 11, $n(t, \tau)$ is plotted for constant t. It is apparent that

$$N(t) = \int_{\tau=0}^{T} n(t,\tau)\,\mathrm{d}\tau$$

which is the area between $\tau = 0$ and $\tau = T$ in Fig. 11.

Defining the mitotic index, $M(t) \times 100$, as the percent of cells in the interval $\tau = 0$ to $\tau = \tau_m$, we have:

$$M(t) = \frac{\int_0^{\tau_m} n(t,\tau)\,\mathrm{d}\tau}{\int_0^{T} n(t,\tau)\,\mathrm{d}\tau} \tag{6}$$

$$\begin{aligned} &= \mathrm{e}^{\alpha\,\tau_m} - 1 \\ &\simeq \alpha\,\tau_m,\ \tau_m \ll T \end{aligned} \tag{7}$$

The mitotic index is constant with time.

Partially synchronous case: To evaluate $n(t, 0)$ for the case where the growth curve is not smoothly exponential, one makes use of eqn. (6):

$$M(t) = \frac{\int_0^{\tau_m} n\,(t,\tau)\,\mathrm{d}\tau}{N(t)} \simeq \frac{n(t,0)\,\tau_m}{N(t)}, \qquad \tau_m \ll T$$

so that $$n(t,0) \simeq \frac{1}{\tau_m} N(t)\, M(t) \tag{8}$$

Using eqn. (2), which still applies, and integrating, the result is obtained:

$$n(t,\,0) = \frac{1}{\tau_m} N_0\, M(t) \exp \frac{1}{\tau_m} \int_0^t M(t)\mathrm{d}t \tag{9}$$

From measured values of $M(t)$, $n(t, 0)$ may be calculated. To evaluate τ_m, the following boundary conditions which arise from the assumption in eqn. (4) are applied:

$$M(t + T) = M(t)$$

$$n(t + T, 0) = 2n(t, 0)$$

From eqn. (9):

$$\tau_m = \frac{1}{\ln 2} \int_0^T M(t) \mathrm{d}t \tag{10}$$

The percentage of cells taking up label

Asynchronous case: Referring to Fig. 11 and using eqn. (4), the percent of labelled cells $L(t) \times 100$ as a function of time t is given by:

$$L(t) = \frac{\int_{G_2}^{G_2 + S + t} n(t,0)\mathrm{d}t}{N(t)}, \text{ for } t < G_2$$

$$= \frac{\int_{G_2}^{G_2 + S + t} n(t,0)\mathrm{d}t + \int_{T + G_2}^{T + t} n(t, 0)\mathrm{d}t}{N(t)}, \text{ for } t > G_2 \tag{11}$$

where label is added at time $t = 0$.

The second integral in eqn. (11) for $t > G_2$ arises from the fact that after time G_2 has elapsed, cells that have picked up label begin to divide, thus increasing the percentage of labelled cells. It is assumed in eqn. (11) that all cells have the same G_2 period. The theoretical treatment is improved by correcting for the division of labelled and unlabelled cells, taking into account the variation in G_2 times. The first labelled cells to arrive in division are spread out in time in the way depicted in Fig. 4 in the text. From the experimental data of Fig. 3 and the observed $L(t)$ values, the percentage of labelled cells which would have been observed had no cell division taken place, can be obtained. It has the form

$$L(t)_{\text{corr}} = \frac{1}{N_0}\left[L(t)_{\text{exper}} N(t) - \int_0^t P(t - \tau_m)\, aN(t)\mathrm{d}t\right] \tag{12}$$

Where $aN(t)\mathrm{d}t$ is the increase in cell number over a time interval $\mathrm{d}t$, and $P(t - \pi_m)$ is the fraction of labelled cells entering division, taken from Fig. 3. Allowance is made for the fact that metaphase precedes division by time τ_m. The first term in the bracket represents the total number of labelled cells that are seen, and the second term represents the number of these that are due to division of already labelled cells.

After a time G_1 has elapsed, a further correction would be required to allow for cells which have divided after addition of label and which have not been in the S period in the presence of label. There is little point in extending the treatment for times larger than G_1, however, since the spread in S and G_1 times would have to be accurately known or the uncertainty introduced in making such a correction would be large.

Thus for $t < G_1$, eqn. (11) becomes:

$$L(t)_{\text{corr}} = \frac{\int_{G_2}^{G_2 + S + t} n(t,0)\mathrm{d}t}{N_0} \tag{13}$$

Using eqn. (3), this gives:

$$L(t)_{\text{corr}} = e^{\alpha G_2}\left[e^{\alpha(s+t)} - 1\right], \qquad t < G_1 \tag{14}$$

From which one obtains:

$$S = \frac{1}{\alpha}\ln\left(L(t)_{\text{corr}} + e^{\alpha G_2}\right) - (G_2 + t) \tag{15}$$

For each corrected experimental $L(t)$ value, a value for S can be computed.

Partially synchronous case: Using the $n(t, 0)$ values obtained from eqn. (9), the experimental $L(t)$ values can be corrected for division by applying eqn. (12) substituting $n(t,0)$ for $\alpha N(t)$. Eqn. (13) still applies, and using $n(t,0)$ again, values of $L(t)_{\text{corr}}$ may be calculated for various values of S, and the S chosen which gives best agreement between calculated and experimental values. The integration is done graphically.

Calculation of average DNA content per cell for an asynchronous exponential culture

Assuming a linear synthesis of DNA over the S period and referring to Fig. 11, the total DNA in the culture at time t is given by the sum of three terms: first, a term for the cells in the G_2 period which all have a DNA content of 2 units (2D), second, a term for the cells in the G_1 period which have a DNA content of 1 unit (1D) and third, a term for those in the S period which have a DNA content ranging linearly from 1 unit to 2 units. The average DNA/cell is thus given by:

$$\frac{\text{Total DNA}}{N(t)} = \frac{1}{N(t)}\left\{\int_0^{G_2} 2\,\mathrm{D}n(t,\tau)\,\mathrm{d}\tau + \int_{G_2+S}^{T} \mathrm{D}n(t,\tau)\,\mathrm{d}\tau + \int_{G_2}^{G_2+S} \mathrm{D}\left[2 + \frac{G_2}{S} - \frac{\tau}{S}\right] n(t,\tau)\,\mathrm{d}\tau\right\} \tag{16}$$

The time (t) dependence naturally drops out of eqn. (16) so that the average DNA/cell is constant with time. If S is 7 h, G_2 4 h and T is 20 h, eqn. (16) gives the average DNA per cell to be 1.30 D or 0.65 the premitotic content.

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CALCIUM CONTENT OF BACTERIOPHAGE T2

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(Received April 10th, 1959)

SUMMARY

1. The calcium content of preparations of bacteriophage T2 has been determined. Each T2 particle contains 130 ± 19 atoms of bound calcium; 40 of the calcium atoms are bound more strongly than the others.

2. The calcium appears to be bound to the tail protein of the intact phage and is released when the tail protein contracts after interaction with host cell walls.

3. There appears to be *one* calcium bound at each ATP binding site of the phage tail protein. It has been concluded that the calcium stimulates the hydrolysis of the ATP during the contraction of the phage tail protein during viral invasion.

INTRODUCTION

The addition of calcium to the growth medium is necessary for the infection of *Escherichia coli* B by either bacteriophages T1[1] or T5[2] but not by T2. In the case of T5, it has been clearly shown that the calcium is not required for adsorption but for the injection of the viral DNA into the host cell[3]. Recently, it has been found that there is a contractile protein in the tail of bacteriophage T2 which contains bound ATP and dATP[4]. Contraction takes place prior to the injection of the DNA. Since calcium stimulates contraction in many systems the possibility has been examined that one of the reactions during T2 invasion may also require calcium but that this calcium is bound in the tail of T2. It has now been found that T2 contains one atom of calcium for each molecule of adenine nucleoside triphosphate.

MATERIALS AND METHODS

Bacteriophage T2L stocks were grown on *E. coli* strain B in liquid medium with vigorous aeration at 37°. The host bacteria were grown to a concentration of $2 \cdot 10^8$/ml and T2 added so that there was 1 phage/bacterium. After infection the suspension was aerated for 6 h, and then chilled, and $CHCl_3$ (2 ml/l) was added to ensure complete lysis. The phage titers of the lysates were usually over $4 \cdot 10^{11}$/ml. The medium was similar to that of FRASER AND JERRELL[5] and contained per l: 20 g glycerol, 10 g casamino acids (Difco), 1.2 g NH_4Cl, 1.0 g NaCl, 0.5 g KCl, 2.4 g Tris, 0.1 g gelatin,

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The abbreviations used in this paper are as follows: DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; dATP, deoxyadenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane. In some instances ATP is used as an abbreviation for both ATP and dATP.

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and various divalent metal ions all adjusted to pH 7.4. In all experiments the medium contained $2 \cdot 10^{-3}$ *M* $MgSO_4$ and 10^{-7} *M* $FeSO_4$, $ZnCl_2$ and $MnCl_2$. No extra calcium was added to the media (except where noted) since the casamino acids contained enough calcium to give a final concentration of approx. 10^{-4} *M*.

^{45}Ca was obtained from the Oak Ridge National Laboratory as $^{45}CaCl_2$ and was determined in an internal flow Geiger counter as infinitely thin samples. T2 was grown in media containing between 0.2 and 0.6 μC of ^{45}Ca/ml. The calcium content of the ^{45}Ca medium used in these experiments was measured by the chloranilic acid precipitation procedure of FERRO AND HAM[6]. When the Ca^{++} concentration was 10^{-4} *M* or less, chloranilic acid precipitated only 25–75 % of the total calcium. This did not interfere with the measurement of the specific activity of the ^{45}Ca since appropriate corrections could be made on the basis of the fraction of radioactivity precipitated. As only 0.1 to 0.01 % of the radioactivity was isolated in the phage, the possibility that the ^{45}Ca was contaminated with other radioactive elements was examined. Contamination was ruled out since the radioactivity in a sample of purified T2 phage grown in media containing ^{45}Ca decayed with the half-life characteristic of ^{45}Ca.

Cell walls were prepared by the procedure of SALTON AND HORNE[7], and P was measured by the method of GOMORI[8].

RESULTS

Calcium content of bacteriophage T2

The calcium content of T2 bacteriophage was determined by growing the virus in medium containing ^{45}Ca of known specific activity, purifying the virus and calculating the Ca content from the radioactivity of the pure virus. One difficulty of determining the calcium content of T2 is the porous structure of the T2 head and the high affinity of the viral DNA within the head for calcium. Calcium was found to penetrate readily the head membrane of intact T2 and to be bound tightly to the DNA*. Since this investigation was concerned with the possible presence of calcium bound to the viral tail protein, calcium inside the head membrane was an undesirable complication. Therefore, in most experiments the calcium concentration of the growth medium was kept low (approx. 10^{-4} *M*) and there was always present $2 \cdot 10^{-3}$ *M* Mg^{++} to compete with Ca^{++} in the binding to DNA. The phage was purified by differential centrifugation in the usual manner; the virus was washed with 0.9 % NaCl containing 0.01 *M* $MgSO_4$ to displace bound calcium. When T2 is grown and purified by these procedures, preparations of essentially constant ^{45}Ca content can be obtained as in Preparation 1 in Table I. The supernatant obtained from the fourth wash of this preparation contained less than 1/15 of the ^{45}Ca in the phage (some of which was due to unsedimented phage).

The values obtained for the calcium content of T2 are given in Table II as atoms of calcium per T2 particle. The number of Ca atoms was calculated from the specific activity of the ^{45}Ca of the growth medium, and the number of virus particles from their phosphorus content. The value of $2.3 \cdot 10^{-11}$ μg P/T2 was used[9] and assays of the T2 preparations gave values approaching closely those based on the phosphorus

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* The conclusion that calcium ions penetrate the head membrane of intact T2 rests on the observation that after incubation of T2 in 1.3 *M* $CaCl_2$, rapid dilution with water causes osmotic shock and the release of the viral DNA.

TABLE I

PURIFICATION OF T2 CONTAINING ^{45}Ca FROM MEDIA OF DIFFERENT CALCIUM CONCENTRATIONS

See text for composition of media and purification procedure.

Preparation I *10^{-4} M calcium in medium*		*Preparation IV* *10^{-3} M calcium in medium*	
Material	*^{45}Ca Per cent*	*Material*	*^{45}Ca Per cent*
Growth media	100	Growth media	100
Supernatant from media approx.	90	Supernatant from media approx.	90
Supernatants from T2 washes		Supernatants from T2 washes	
1st wash	1.9	1st, 2nd, 3rd, and 4th	4
2nd wash	0.6	5th	0.12
3rd wash	0.05	6th	0.12
4th wash	0.01	7th	0.006
		8th	0.003
T2 after 4 washes	0.15	T2 after 4 washes	0.067
		T2 after 6 washes	0.017
		T2 after 8 washes	0.009

TABLE II

CALCIUM CONTENT OF BACTERIOPHAGE T2

Preparation	*Divalent cations in media*			*Atoms of calcium per T2 particle**	
	Ca^{++} M	*Mg^{++} M*	*Mn^{++} M*	*Purified by centrifugation***	*After dialysis against citrate***
I	10^{-4}	$2\cdot10^{-3}$	10^{-7}	142 ± 20	42
II	10^{-4}	$2\cdot10^{-3}$	10^{-7}	107 ± 15	32
III	10^{-4}	$2\cdot10^{-3}$	10^{-3}	140 ± 20	—
IV	10^{-3}	$2\cdot10^{-3}$	10^{-7}	<400	40

* Calculated from the ^{45}Ca content and the phosphorus content.
** See text for details of the purification procedure.

content. The average value found for 3 preparations when T2 was grown in medium containing 10^{-4} *M* $CaCl_2$ was 130 ± 19 Ca atoms/T2 particle. This number is identical to the 131 ± 10 molecules of adenine nucleoside triphosphates found in the T2 particle[4].

When T2 was grown in media containing 10^{-3} *M* $CaCl_2$ it was difficult to measure the number of calcium atoms per T2 particle (Table II). As illustrated in Table I, significant amounts of calcium could be removed by continued washings in the centrifuge. For example, in Preparation IV, after 4 washings there were 2200 Ca atoms/T2, after 6 washings 658 atoms/T2 and after 8 washings 400 Ca atoms/T2. But even after the eighth washing in the centrifuge, the supernatant contained 1/3 the amount of calcium associated with the phage. Clearly in Preparation IV all the calcium cannot be considered an integral part of the viral structure.

Samples of purified T2 from Preparations I, II and IV were dialysed for 5 days at 6° against daily changes of 100 volumes of 1 % sodium citrate at pH 7.4*. The number of Ca atoms remaining per T2 particle was measured in terms of the P content. Approx. 40 Ca atoms remained firmly bound to each virus particle (Table II) and could not be removed by this treatment*. Presumably the location of these 40 Ca atoms in the virus structure does not permit their removal. This observation is similar to that made in studies on the location of the adenine nucleoside triphosphates in T2. About 40 (of the 130) nucleotides appear to be bound within the tail structure and cannot be extracted by cold acid. These similarities support the conclusion that the calcium found in the T2 preparations is not an adventitious contaminant.

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* The virus is inactivated by this treatment.

The incorporation of 130 calcium atoms into the T2 structure appears to involve quite specific bonds since the calcium content of T2 was not affected by the presence of 10^{-3} $MnCl_2$ in the growth medium (Preparation III). The effect of the presence of Mn^{++} was investigated because Mn^{++} can replace Ca^{++} in allowing T5 injection and is even more effective than Ca^{++} in stimulating the ATPase activity of T5[10]. Further, Mn^{++} was found by BAILEY[11] to be the only ion besides Ca^{++} which stimulates myosin ATPase.

Treatments affecting the ^{45}Ca content of purified T2 preparations

The effect of various treatments on the calcium content (and the nucleoside triphosphate content) of purified T2 is shown in Table III. When T2 is added to a preparation of cell walls of *E. coli* the virus is adsorbed, the tail contracts and the viral ATP (and dATP) is broken down. It can be seen that most of the viral ^{45}Ca is released from the T2 after interaction with cell walls and appears in the medium. Similarly, after the tail of the virus particle is treated with $Cd(CN)_3^-$ the tail contracts[4] and most of the ^{45}Ca and ATP are found to be no longer bound to the phage particle.

A considerable fraction of the ^{45}Ca exchanges readily with externally added Ca^{++} and Mn^{++} but not with Mg^{++}. The exchange with Mn^{++} appears to be even faster than with Ca^{++}. Phage equilibrated with 0.08 *M* $MnCl_2$ under conditions permitting maximal ^{45}Ca replacement was still completely infective in the usual assay procedure. It seems possible that although Ca^{++} is always incorporated into the T2 phage structure, Mn^{++} can substitute for calcium in invasion by T2 as it does with T5.

TABLE III

TREATMENTS REDUCING CALCIUM CONTENT OF T2

All treatments were carried out at 37° for 2 h. There were 50 T2 particles/cell wall in 0.01 *M* tris buffer pH 7.5 and 0.5 *M* NaCl. The $Cd(CN)_3^-$ was prepared as described earlier[12]. The T2 was equilibrated with the metal ions in 0.01 *M* tris buffer pH 7.5, and then centrifuged down at high speed.

Treatment	*Atoms of calcium per T2*			*ATP per T2**
	Prep. I	*Prep. II*	*Prep. III*	
1. None	142	107	140	131
2. Interaction with cell walls	40	30	12	40
3. Alteration of tail by $Cd(CN)_3^-$	40	10	15	22
4. Equilibration with 0.08 *M* $CaCl_2$	37	57	70	—
5. Equilibration with 0.08 *M* $MgSO_4$	120	95	130	—
6. Equilibration with 0.08 *M* $MnCl_2$	40	42	40	—

* See [4], the values given represent both ATP and dATP.

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* Control experiments in which purified unlabeled T2 was equilibrated in ^{45}Ca (in the absence of host cells) were carried out. The phage was then purified by centrifugation and dialyzed against citrate. After this treatment the preparations contained only 2 or 3 calcium atoms/T2 particle.

In all exchange reactions there appears to be a residual amount of unexchangeable Ca^{++} which approached 40 Ca atoms per T2 particle. This supports the view that 40 of the binding sites for Ca^{++} (and ATP) in the tail are especially strong.

DISCUSSION

The validity of the estimation of minor components in preparations of T2 bacteriophage particles depends on the evidence that the component is not a contaminant, and acquires significance if a physiological function can be assigned to the component. In view of the high affinity of DNA for calcium purification to the same constant calcium content for different phage preparations suggests but does not conclusively show that the calcium is an essential viral component. Support for the view that the calcium found in these T2 preparations is indeed a genuine component comes (a) from considerations of the properties of T2 as compared to other phage particles; (b) from the amount of calcium found; and (c) from the types of treatments which reduce the calcium content of the phage particles. It is well known that the 2 thin-tailed bacteriophages T1 and T5 require the presence of calcium to inject their DNA into the host *E. coli*[1-3]. T2 does not require additional calcium presumably because it contains a sufficient amount of calcium bound within its relatively thick tail.

T2 possesses a protein in its tail which contracts prior to the injection of the phage DNA into the host cell[4]. The present experiments suggest that the calcium found in phage preparations is associated with this contractile protein. The contractile protein of one phage particle binds 131 molecules of adenine nucleoside triphosphate, 40 of which are bound more tightly than the rest. The present experiments show that T2 contains 130 atoms of calcium, 40 of which are bound much more strongly than the others. When the phage tail contracts either after interaction with cell walls or treatment with $Cd(CN)_3^-$ it no longer binds either ATP or calcium. It seems very likely that there is one Ca atom at each ATP binding site on the T2 tail. In view of the similarities between the contractile protein of T2 and actomyosin[4] whose ATPase activity is stimulated by Ca^{++}, and of the fact that the ATPase of T5 is stimulated by Ca^{++} it seems likely that the calcium stimulates the rate of breakdown of ATP so that contraction of the viral tail protein can occur as rapidly as possible.

ACKNOWLEDGEMENTS

This work was aided by grants from the National Foundation for Infantile Paralysis, Inc., and the Dr. Wallace and Clara A. Abbott Memorial Fund at the University of Chicago.

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THE INTERACTION OF POLYNUCLEOTIDES WITH METAL IONS, AMINO ACIDS, AND POLYAMINES

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(Received April 21st, 1959)

SUMMARY

The formation of the two-stranded complex between the sodium salts of polyadenylic acid and polyuridylic acid requires the presence in solution of additional cations. It is found that the effect of magnesium ion is characteristic of a strong interaction between metal ion and polynucleotide, while the effect of sodium ion is characteristic of weak interaction.

It is also found that the formation of the two-stranded structure requires neutralization of the charge on the phosphate backbone. This information makes it possible to use the extent of the poly A–poly U interaction as a measure of binding of cations to polynucleotide. The method is applied to charged amino acids and polyamines. It is shown that the amino acids are weakly bound and the polyamines strongly bound to polynucleotides.

INTRODUCTION

The interaction of the synthetic polyribonucleotides, polyadenylic acid and polyuridylic acid, with each other is dependent upon the nature and concentration of cations present in the solution[1–3]. Previous studies[4] have shown that both poly A and poly U interact strongly with Mg^{++} and Mn^{++} and that about one equivalent of divalent ion is bound per mole of phosphate. In this paper we describe further studies of the ionic conditions necessary for the combination of poly A and poly U, and use this information to provide a simple measure of the strength of interaction between polynucleotides and both charged amino acids and polyamines.

EXPERIMENTAL

The polynucleotides were prepared enzymically from nucleoside diphosphates with polynucleotide phosphorylase isolated from *Azotobacter agile* according to a method kindly provided by Prof. S. OCHOA. Preparations were dialyzed in succession against ethylenediaminetetra-acetic acid, sodium chloride, and redistilled water before freeze-drying. Experiments involving mixing of poly A and poly U were performed so that the pH of the final reacting mixtures was generally between 6.7 and 7.4. Where necessary the pH of solutions was adjusted with NaOH, and the reacting mixtures of polymer were stored in dessicators over saturated NaOH solution to eliminate absorption of atmospheric CO_2. These precautions were necessary to avoid the formation of the ordered poly A complex[5,6] which occurs in distilled water below pH 6.5. The upper limit in pH is necessary to avoid metal ion–hydroxyl ion interactions in certain cases.

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* Contribution No. 64 from the Department of Biophysics.

Abbreviations: Polyadenylic acid = poly A; polyuridylic acid = poly U; deoxyribonucleic acid = DNA; ribonucleic acid = RNA. The materials referred to by these names are the sodium salts.

The amino acids were commercial preparations (glycine, Fisher; L-histidine, California Corporation for Biochemical Research; L-histidine monohydrochloride, Eastman; imidazole, Eastman; L-lysine monohydrochloride, Interchemical). Putrescine hydrochloride was obtained from California Corporation for Biochemical Research, and was analyzed for chloride ion by silver nitrate titration. Diaminodecane and diaminopentane (cadaverine) were obtained from Aldrich Chemical Company, and were analyzed for titratable amino groups by dissolving weighed quantities in an excess of standard hydrochloric acid and back-titrating with standard sodium hydroxide solution. The diaminopentane and diaminodecane were also analyzed for nitrogen colorimetrically with Nessler's reagent. The results agreed with the titration within 3 %.

Solutions of sodium, manganese and magnesium chloride were made from analytical grade reagents. The divalent ion chlorides were standardized by titration with silver nitrate using dichlorofluorescein as an indicator.

O.D. measurements were made with the Cary Model 14 recording spectrophotometer. All O.D. reported were measured at 259 mμ. In the case of the most dilute solutions, cylindrical cells with a path length of 10 cm were used. In the case of the most concentrated solutions, rectangular cells of path length 0.5 cm were used, and when the O.D. exceeded 2.0, readings were made against standardized blanks of sufficiently high O.D. to bring the readings within the range of the spectrophotometer.

Ultracentrifuge studies were made with the Spinco Model E, equipped with u.v. optics.

RESULTS

Metal ions

The decrease in O.D. in the 260-mμ region which accompanies the combination of poly A and poly U in solution has already been used to obtain qualitative information about the relative effectiveness of various cations in promoting the combination[2]. In order to relate the fractional decrease in O.D. to the fractional completion of the reaction, it is naturally necessary to establish that the entire drop in O.D. which occurs when cations are added is attributable to the reaction. Investigation of the effect of the addition of $MgCl_2$ on the O.D. of poly A alone shows that this is not the case (Fig. 1). With the pH carefully adjusted above 7.0 so that the "ordered" poly A structure[5,6] is unstable, poly A shows a marked hypochromic effect upon addition of small amounts of divalent ion. The molar O.D. is independent of polymer concentration for a given ratio of equivalents of divalent ion added to moles of polymer phosphate, and the limiting O.D. is reached when about one equivalent of divalent ion has been added/mole of polymer phosphate. The total hypochromic effect is about

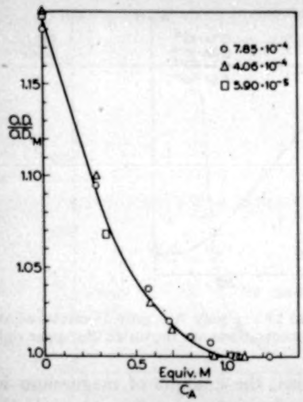

Fig. 1. O.D. (divided by limiting O.D.) of poly A solutions at various concentrations plotted against equivalents of magnesium ion added/mole of polymer phosphate. Measured at 259 mμ.

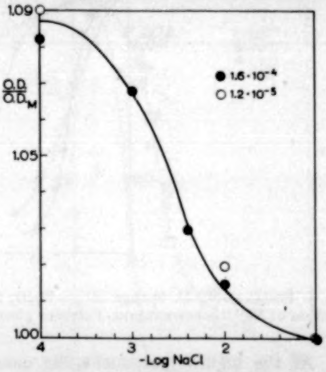

Fig. 2. O.D. (divided by limiting O.D.) of poly A solutions at two concentrations plotted against the negative logarithm of NaCl concentration.

16 % of the O.D. in distilled water. The limiting molar O.D., about 8840 at 259 mμ, is somewhat lower than that obtained in 0.1 M NaCl at pH 6.7[2].

Poly U does not show such strong dependence of O.D. upon ionic concentration. A small decrease of about 3 % in O.D. is observed in going from distilled water to one equivalent of divalent ion.

The effect of the addition of sodium chloride to poly A in distilled water is characteristically different from that obtained with divalent ions. Though there is a drop in O.D. (Fig. 2), the change occurs much more gradually as the salt concentration is increased, beginning at 10^{-3} M NaCl, but not complete until the concentration is raised to 10^{-1} M, so that NaCl is in large excess. The total drop in O.D. is only about half that observed when divalent ions are added.

With the use of these corrections it is possible to interpret the O.D. decrease of a mixture of poly A and poly U in terms of formation of poly (A + U). If we assume that the O.D. contributed by partly formed poly (A + U) is directly proportional to the number of combined sites, it is easy to show that F, the fraction of completion of combination, is given by

$$F = \frac{D'_0 - D}{D'_0 - D_M} \qquad (1)$$

where D is the O.D. of the poly A + poly U solution in question. D'_0 is the sum of the O.D. of two solutions containing poly A and poly U separately at the concentration used in the mixed solution, and with the same amount of salt added, and D_M is the limiting O.D. density of the mixture at high salt concentration.

In Fig. 3 $(D'_0 - D)/D_0$ is plotted as a function of magnesium ion concentration for a series of 1 : 1 poly A + poly U mixtures over a hundred-fold range of polynucleotide concentration. With a knowledge* of D_0, it is possible to compute F.

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* The molar O.D. at 259 mμ in distilled water is about 11,600 for poly A and 9700 for poly U.

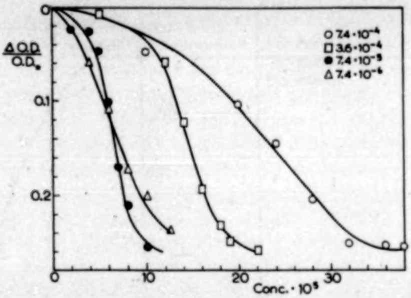

Fig. 3. Fractional O.D. change $(D'_0 - D)/D_0$ for a series of 1 : 1 poly A – poly U mixtures as a function of Mg^{++} concentration. Polymer phosphate concentrations are shown at the upper right.

At the highest polynucleotide concentrations, the amount of magnesium ion necessary to cause poly A and poly U to interact increases as the polynucleotide concentration increases. Within about 10 %, the reaction requires one equivalent of divalent ion/mole of phosphate to go to completion. At the lowest concentration there appears to be a limiting curve. Similar experiments involving the addition of Mn^{++} rather than Mg^{++} give the same results.

The addition of NaCl to a 1 : 1 poly A – poly U mixture also causes combination of the two polymers, but in this case the degree of reaction for a given sodium chloride concentration is independent of polynucleotide concentration (Fig. 4). As in the case of the O.D. drop of poly A alone, about a 100 fold increase in NaCl concentration is required to go from $F = 0.01$ to $F = 0.99$.

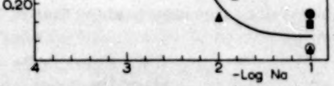

Fig. 4. Fractional O.D. change $(D'_0 - D)/D_0$ for a series of 1 : 1 poly A – poly U mixtures as a function of the negative logarithm of NaCl concentration. Polymer phosphate concentrations are shown at the upper right.

Amino acids and diamines

Having established the behavior of two classes of ions, those which are bound strongly and those which are bound weakly (see DISCUSSION), we can use the poly A – poly U interaction as a measure of cationic binding.

In Fig. 5 are shown the effects upon the O.D. of a 1 : 1 poly A – poly U mixture of the addition of various amino acids, compared with the effect of NaCl upon the

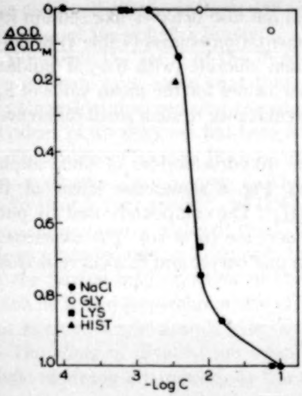

Fig. 5. Decrease in O.D. divided by maximum decrease in O.D. (uncorrected for changes in poly A alone) plotted against the negative logarithm of the molar concentration of hydrochlorides of glycine, lysine and histidine, compared with the effect of NaCl. Polymer phosphate concentration = $7.2 \cdot 10^{-5}$.

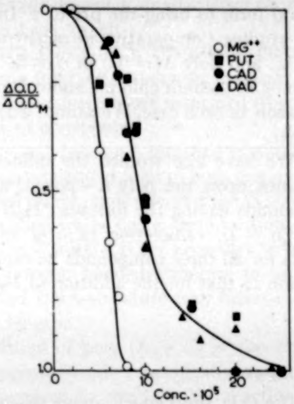

Fig. 6. Decrease in O.D. of poly (A + U) divided by maximum decrease in O.D. (uncorrected for changes in poly A alone) plotted against concentration of putrescine, cadaverine and diaminodecane hydrochlorides, compared with the effect of $MgCl_2$. Polymer phosphate concentration = $6.7 \cdot 10^{-5}$.

same solution. Glycine, which has no net charge, produces essentially no O.D. change even in 1 *M* solution. Positively charged lysine, (lysine hydrochloride) on the other hand, does cause an interaction to exactly the same extent as sodium ion at the same concentration.

In the case of histidine it is necessary to add a mixture of the free amino acid and the hydrochloride to keep the pH at 7 or above, since at lower pH there is danger of formation of the ordered, non-reactive poly A structure. Because the p*K* of histidine is 6.05, approximately a ten-fold excess of neutral histidine must be present in solution to keep the pH at 7. Since glycine does not cause poly A and poly U to interact, we assume that no other dipolar ion has the ability to do so. The concentration of positively charged histidine is computed from the measured pH and the total histidine concentration (assuming that a negligible amount is bound to the polymer). When the O.D. drop is plotted against the negative logarithm of concentration of the charged histidine species, it is found that these points also fall on the curve for sodium chloride.

The effect of imidazole has been studied in a similar manner, though in this case the p*K* of the ring nitrogen is higher, so that only half the imidazole need be in the

TABLE I

S_{20}	*0.05 M NaCl*	*0.05 M imidazole hydrochloride + 0.05 M imidazole*
Poly A	11.0	11.4
Poly U	5.7	5.9
Poly (A + U)	8.9	9.2

neutral form to bring the pH to 7. Imidazolium ion also behaves like sodium ion in O.D. studies. Comparative ultracentrifuge sedimentation studies (Table I) of poly A, poly U, and poly A(+ U) in 0.05 *M* imidazolium chloride (with 0.05 *M* imidazole) and 0.05 *M* sodium chloride show slightly higher values for the mean value of S_{20} in imidazole in each case. We cannot attach any significance to such small differences at present.

We have also studied the influence of the dihydrochlorides of some aliphatic diamines upon the poly A – poly U interaction. Fig. 6 shows the effect of three compounds having the formula $^{+}H_3N(CH_2)_nNH_3^{+}$. The compounds used are putrescine (n = 4), cadaverine (n = 5) and diaminodecane (n = 10). The experimental points for all three compounds lie essentially on one curve, and this curve is close in position to that for the addition of Mg^{++}.

DISCUSSION

Metal ions

The O.D. drop which occurs when Mg^{++} and Na^{+} are added to poly A cannot be explained by the formation of the "ordered" poly A structure of FRESCO AND DOTY[5] and BEERS AND STEINER[6], since the formation of that structure is reported to be inhibited by the addition of salts, particularly those of divalent ions. In addition, the O.D. maximum in our experiments remains at 257 mμ as salts are added. This is the position of the maximum reported for the "random coil" form of poly A. We do not observe the shift of the maximum to 252 mμ which should occur if the more rigid poly A structure were being formed. The work of FRESCO AND DOTY[5] indicates that poly A in 0.1 *M* NaCl at pH 7.5 is a random coil, so that, in the case of addition of NaCl to poly A, we can be reasonably certain that the hypochromic effect does not involve formation of any multiple-stranded poly A structure. It appears that the hypochromic effect is in this case attributable to the increased flexibility* of the single-stranded poly A molecule as it is neutralized by addition of excess sodium ion. The increased flexibility may lead to more satisfactory opportunities for adjacent bases on a given chain to "stack", and this may in turn increase the hypochromic effect[7].

The same argument applies to divalent ion, except that the neutralization occurs upon the addition of equivalent amounts of ion. The fact that the hypochromic effect is larger for Mg^{++} than for Na^{+} may be explained if the "random coil" is more compact, with better opportunity for stacking, in the presence of the very low magnesium chloride concentrations than it is in the presence of the rather high sodium chloride concentrations which are necessary to reduce the charge on the polymer chain. Measurements of the radius of gyration in each case have not yet been made.

It should be pointed out that there are other explanations of the hypochromic effect which do not involve formation of multiple strands. Dr. E. P. GEIDUSCHEK (personal communication) has suggested that the polarization of the bases in the field of the phosphate ions is largely abolished when the backbone is neutralized, and that this could account for the change in absorption of the bases, and all of the above results. Either hypothesis will also account for the much smaller effect observed in the case of the polymer involving the smaller base, uracil.

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* Polyelectrolytes in general including poly U (H. EISENBERG AND G. FELSENFELD, unpublished results) show a dramatic drop in viscosity when NaCl is added to a distilled water solution.

Whatever the cause of the hypochromic effect in poly A, the results shown in Fig. 1 indicate once again that there is a strong interaction between divalent ions and polynucleotides, and that the sites on the polynucleotide are saturated when one equivalent of divalent ion has been added/mole of phosphate.

The results of SHACK, JENKINS AND THOMPSETT[8] bear an interesting resemblance to those reported here for poly A. These investigators added salts of both monovalent and divalent ions to solutions of DNA in distilled water, and observed the O.D. drop. It was subsequently realized[9] that DNA in distilled water has a large portion of its hydrogen bonded system disrupted. In view of our results, it is not necessary to postulate the partial reconstitution of the DNA hydrogen bonded structure to account for the increased hyperchromicity. The disrupted DNA structure may behave somewhat like two single strands over considerable lengths.

The effect of divalent ion upon the formation of poly (A + U) is also characteristic of strong interaction. At the highest polynucleotide concentrations one equivalent of divalent ion/mole of phosphorus is required to complete the reaction. The excess concentration of divalent ion required to complete the reaction at lower polynucleotide concentrations, and the existence of a limiting curve, suggest a partial dissociation of the poly (A + U) – Mg^{++} complex at these low concentrations.

It is not possible to account quantitatively for the results of Fig. 3 on the basis of any simple reaction scheme of the kind which was used[2] in the study of three-stranded poly (A + 2U). The widely-varying conditions of ionic strength in the experiments described here are sufficient to explain this difficulty. On the other hand, any one of a number of reaction schemes involving partial dissociation of the poly (A + U) – magnesium complex at low concentrations will qualitatively account for the results. Despite the partial dissociation of the complex at low concentrations the interaction is a "strong" one (*i.e.*, the reaction goes to completion with a very small excess of Mg^{++}) at phosphate concentrations of 10^{-4} *M* and above. It should be pointed out that partial dissociation of the poly (A + U) – metal complex is not necessarily inconsistent with a stronger affinity of single polynucleotide strands for divalent ion, since the unit which combines with divalent ion in the two-stranded case may be an uncombined, but neighboring, pair on a partly-formed two-stranded structure. This structure could be somewhat less flexible than the single-stranded polymers, with a consequent decrease in affinity for metal ion[10].

Unlike divalent ions, the effect of NaCl upon the polynucleotide combination is characteristic of a weak interaction. Both the large excess of ion required and the absence of polynucleotide concentration dependence in Fig. 4 are consistent with this interpretation.

Amino acids and polyamines

The contrast between the effects of "strong" and "weak" cation interaction on formation of two-stranded poly (A + U) provide a useful method for studying cation binding to polynucleotides. This is particularly true in the case of strong binding, which can be studied at low ionic strength, thus providing complementary information to that obtained by means of dialysis equilibrium at high ionic strength. At high ionic strength, there is competition for the polynucleotide sites from the sodium ion or other cation used to raise the ionic strength. At low ionic strength the interaction between the cation of interest and the polynucleotide is enhanced by the absence of this competition.

The application of this technique to amino acid binding shows conclusively that even under optimal conditions, charged amino acids are not bound in a selective manner to the polynucleotides studied. Earlier studies[11–13] of the binding of amino acids to DNA and RNA at high ionic strength have resulted in the same conclusion. It is particularly interesting that the large base of the histidine molecule, which might be expected to stabilize the binding through "stacking", does not give histidine any advantage.

The diamines on the other hand are obviously bound strongly to the polynucleotides. The fact that slightly higher concentrations of the diamines than of Mg^{++} are required to cause the poly A – poly U interaction suggests that the diamines are a little less strongly bound than the metal ion, but the affinities are certainly of the same order of magnitude, and it is likely that diamines would compete strongly with divalent ions for sites on polynucleotide chains under conditions of equal concentration, even at higher ionic strength. Naturally, the presence of monovalent ions such as Na^+ under physiological conditions tends to reduce the absolute amount of diamine bound, but in systems which involve diamine concentrations of the order of divalent ion concentration, as much diamine as divalent ion will be bound.

Preliminary studies of binding of putrescine to poly U by means of conductometric titration show that there is also strong interaction between single-stranded polynucleotides and diamines. Other titrations involving the tetra-amine spermine indicate that in distilled water spermine tetrahydrochloride is capable of displacing divalent barium stoichiometrically from poly U, precipitating a spermine–poly U complex. Thus, it appears that in distilled water the affinity of single-stranded polynucleotides for polyamines is strong, and it is probable that the affinity increases as the number of amine groups per molecule increases.

In a physiological situation such as that encountered by the DNA of certain bacteriophages[14], which involves high polyamine concentrations, it is likely that nucleic acids will carry significant amounts of polyamine. The quantity which is carried will of course depend upon the particular polyamines present, their concentration, and the concentration of small ions which can compete for sites on the nucleic acid. In addition, the presence of strongly bound protein chains may block the combination with polyamines.

Our preliminary experiments with spermine indicate a behavior strongly resembling that of polylysine[4]. If the more highly charged polyamines do approach polylysine in their affinity for polynucleotide, they may exert a marked influence upon the physical properties of the polynucleotide to which they are attached. This possibility must be considered whenever a nucleic acid has been isolated from an environment containing polyamines.

ACKNOWLEDGEMENTS

We are indebted to Dr. B. AMES for helpful discussions. This work was supported by Grant C-3883, National Cancer Institute, Public Health Service.

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STUDIES ON THE ENZYMIC OXIDATION OF AMINOPURINES

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(Received April 24th, 1959)

SUMMARY

1. 8-Aminopurine is oxidised by mammalian xanthine oxidase along the following pathway:

8-Aminopurine ⟶ 6-hydroxy-8-aminopurine ⟶ 2,6-dihydroxy-8-aminopurine

It is thus evident that oxidation of all three aminopurines takes a course different from the corresponding hydroxypurines.

2. Adenine and 6-methylaminopurine are oxidised along the same pathway, but all other methyl derivatives of adenine are refractory.

3. 2-Aminopurine is converted by a bacterial enzyme system into 2-amino-8-hydroxypurine.

These results are discussed in terms of specific enzyme–substrate complexes.

INTRODUCTION

Wyngaarden and Dunn[1] have recently established that adenine is attacked by mammalian xanthine oxidase (XO) first at position 8 and thereafter at carbon atom 2. The order of steps is thus at variance with the sequence, applying to the enzymic oxidation of hypoxanthine:

Hypoxanthine ⟶ xanthine ⟶ uric acid
Adenine ⟶ 8-hydroxyadenine ⟶ 2,8-dihydroxyadenine.

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* The results of this investigation are parts of Ph. D. theses, submitted to the Faculty of Science, The Hebrew University, Jerusalem.

Analogously, 2-aminopurine is converted into guanine, as shown by LORZ AND HITCHINGS[2] and the present authors[3], in contrast to the behavior of 2-hydroxypurine:

2-Hydroxypurine ⟶ 2,8-dihydroxypurine ⟶ uric acid
2-Aminopurine ⟶ guanine (= 2-amino-6-hydroxypurine)

In the present study, we find that 8-aminopurine (I) too differs characteristically from 8-hydroxypurine in its behavior towards XO:

8-Hydroxypurine ⟶ 2,8-dihydroxypurine ⟶ uric acid[4]
8-Aminopurine ⟶ 6-hydroxy-8-aminopurine ⟶ 2,6-dihydroxy-8-aminopurine

Thus, a systematic difference exists between these two series of purines, which may be due either to a common structural difference, responsible for the specific electron distribution and the polarity in each group, or to a different mode of attachment of hydroxy and amino derivatives to the active center of the enzyme. An answer to this problem was sought by testing the oxidation of aminopurines by a bacterial enzyme system, described previously[5].

In the formation of the enzyme–substrate (ES) complex, hydrogen bonding plays a decisive role. The groups participating in the binding of adenine to XO have been determined by systematically substituting methyl for hydrogen at all available nitrogen atoms. The results obtained give some information on the "active form" of this substrate, actually involved in the enzymic process.

MATERIALS AND METHODS

Purine derivatives

6-Hydroxy-8-aminopurine[6] was a gift of Dr. R. K. ROBINS, Arizona State College, Tempe, Arizona. 2,6-Dihydroxy-8-aminopurine[7] was obtained through the courtesy of Dr. A. BENDICH, the Sloan-Kettering Institute for Cancer Research, New York. 6-Methylamino- and 6-dimethylaminopurine were synthetic products, prepared by Dr. G. B. ELION of the Wellcome Research Laboratories, Tuckahoe, New York[8]. Likewise, 9-methyladenine[9] was supplied by Dr. ELION. Finally, 7-methyladenine[10] was a gift from Dr. E. C. TAYLOR, Princeton University.

Enzymes

Highly purified milk xanthine oxidase was supplied by Prof. F. BERGEL and Dr. R. C. BRAY of the Chester Beatty Institute of Cancer Research, London, England[11]. This preparation, when diluted 1:800, produced 1 μg/ml of uric acid/min, when the reaction was carried out at 28° and pH 8.0 with $6.5 \cdot 10^{-5}$ M xanthine as substrate.

As source of the bacterial enzyme system, whole cells of *Pseudomonas aeruginosa* BDH, an air-borne strain described previously[5], were used. Identical results were obtained with resting cells or with a culture growing in a liquid medium[12]. However, growing cells exhibited a faster turnover and thus facilitated analysis of the oxidation products. They were therefore used preferentially.

Methods of analysis

The progress of the reactions with mammalian XO was followed spectrophotometrically, using a Beckman thermospacer for temperature control. With the bacterial system, this method is applicable only for fast reactions since the cells slowly secrete a greenish pigment, which exhibits unspecific absorption. At suitable intervals, samples of the bacterial reaction mixtures, kept at 30°, were centrifuged at 3000 rev./min and the supernatant used for spectral measurements.

All runs were submitted to paperchromatographic analysis. After development, the individual spots were located by means of their fluorescence under a Mineralight u.v. lamp, emitting light of about 255 mμ wave length. They were then extracted with water and their spectrum measured. When necessary, the shift of λ_{max} with pH was also determined (see under RESULTS).

RESULTS

Reaction of 8-aminopurine with mammalian xanthine oxidase

Preliminary experiments indicated that oxidation of (I) passes through the 6-hydroxy derivative (II). The formation of the alternative intermediate, *viz.* 2-hydroxy-8-aminopurine—which is unknown—, would have been recognised easily by a temporary shift of the absorption maximum to higher wave lengths, because all 2,8-disubstituted purines exhibit maxima above 300 mμ. Fig. 1 shows the absorption spectra of (I), the intermediate (II) and the end-product (III). At 276 mμ, the isosbestic point of (II) and (III), further oxidation of the intermediate produces no change. Therefore, the initial decrease of optical density (O.D.) at this wave length

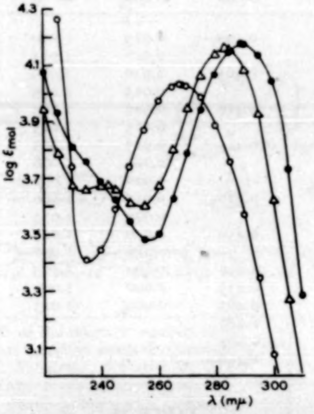
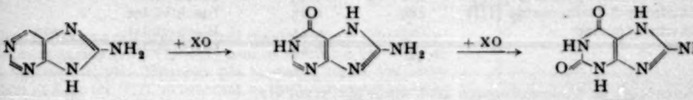


Fig. 1. U.v. absorption spectra of 8-aminopurine and its oxidation products at pH 8.0. △——△ 8-Aminopurine (I). ○——○ 6-Hydroxy-8-aminopurine (II). ●——● 2,6-Dihydroxy-8-aminopurine (III).



(I) (II) (III)

measures the first oxidation step (see Table I). The experimental data also demonstrate that at 273 mμ, the isosbestic point of (I) and (II), the O.D. remains constant for the first half hour and decreases only later, when sufficient intermediate has accumulated to give measurable rates of formation of (III). At 260 mμ, the O.D. increases for the first one and a half hour, as required by the curves in Fig. 1, and then again decreases. The reverse behavior is found at 290 mμ. All data taken together establish beyond doubt that (II) is the main, if not the only intermediate in the enzymic oxidation of (I). The rate of the conversion (I) ⟶ (II) is compared in Table II with that of the second step, (II) ⟶ (III), which was determined at 268 and 290 mμ. The latter conversion proceeds at a rate more than 10 times higher than that of the first step, when equimolar substrate concentrations are used.

TABLE I

SPECTRAL CHANGES DURING THE OXIDATION OF 8-AMINOPURINE

Substrate: $7.4 \cdot 10^{-5}$ *M*; XO, 1:100; 10^{-3} *M* phosphate buffer, pH 8.0. Temperature 28°. All readings represent the difference between this experiment and a second vessel, containing only substrate and buffer. Catalase, 1:500, was added to both vessels.

Time min	*Change of O.D. at*			
	260 mμ	*273 mμ*	*276 mμ*	*290 mμ*
1	0.400	0.915	1.090	1.095
3		0.920	1.085	1.090
5	0.405	0.920	1.080	1.085
7		0.915	1.075	1.075
9	0.410	0.920	1.065	1.080
11		0.915	1.065	1.075
13	0.415	0.915	1.065	1.065
15		0.920	1.068	1.062
17		0.920	1.062	1.060
19	0.420	0.915	1.060	1.050
22		0.920	1.035	1.040
25	0.430	0.910	1.025	1.018
30		0.915	1.018	1.020
40	0.440	0.900	1.025	1.025
55	0.453	0.890	1.020	1.032
70	0.465	0.900	1.015	1.040
95	0.480			1.060
140	0.465			1.085
195	0.440			1.200

TABLE II

CHARACTERISATION OF 8-AMINOPURINE AND ITS OXIDATION PRODUCTS

Substance	λ_{max} *at pH 8.0 (mμ)*	R_F *value in solvent A*[*]	*Fluorescence*	*Relative rate*[**] *of oxidation (xanthine = 100)*
8-Aminopurine	243 283	0.71	Blue-violet	1.3
6-Hydroxy-8-aminopurine (II)	268	0.43	Black-violet	18
2,6-Dihydroxy-8-aminopurine (III)	289	0.25	Black-violet	
6,8-Dihydroxypurine	261	0.45	Black-violet	100
Uric acid	292	0.26	Black-violet	

[*] Solvent A: 95% alcohol–acetic acid–water (85:5:10) v/v.

[**] All substrates were used at 6.5–$7.5 \cdot 10^{-5}$ *M*; XO, 1:800; pH 8.0.

Chromatographic analysis revealed the presence of 3 spots after a reaction period of 1 h (Fig. 2). All 3 components were identified by absorption measurements. 6-Hydroxy-8-aminopurine (II) is clearly distinguished from 6,8-dihydroxypurine by its λ_{max}, although the R_F values in the solvents used are practically identical (see Table II). On the other hand, the absorption maximum of (III) is close to that of uric acid and the same is true for the R_F values of this pair. Since purified XO may include occasionally small amounts of desaminases[13], it was deemed necessary to prove that during the oxidation of (I) the amino group had not been lost. Unequivocal evidence for the structure of (III) could be obtained by following the shift of λ_{max} with pH changes. As shown in Fig. 3, the most characteristic difference between (III) and uric acid appears in the acid range, because only the former can give a cation in acid solution, whereas the absorption maximum of uric acid does not change below pH 4.

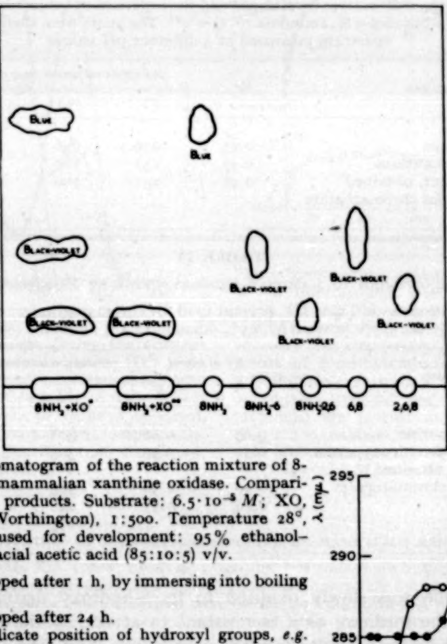


Fig. 2. Paper chromatogram of the reaction mixture of 8-aminopurine with mammalian xanthine oxidase. Comparison with synthetic products. Substrate: $6.5 \cdot 10^{-5}$ *M*; XO, 1:100; catalase (Worthington), 1:500. Temperature 28°, pH 8.0. Solvent used for development: 95% ethanol–water–glacial acetic acid (85:10:5) v/v.

* Reaction stopped after 1 h, by immersing into boiling water for 10 min.

** Reaction stopped after 24 h.

(Numbers indicate position of hydroxyl groups, *e.g.* 6,8 = 6,8-dihydroxypurine)

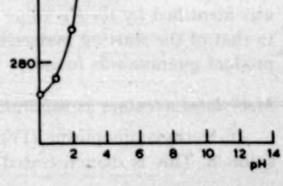


Fig. 3. The long-wave absorption maxima of 2,6-dihydroxy-8-aminopurine (III) (○—○) and of uric acid (●—●) as a function of pH. Between pH 0 and 4, note the large shift of λ_{max} for (III), in contrast to the unchanged position of the absorption maximum of uric acid.

Oxidation of aminopurines by a bacterial enzyme system

8-Aminopurine was not attacked during 72 h of incubation at 30°. Adenine, on the other hand, underwent a slow change. When the supernatant of the bacterial reaction mixture was analysed chromatographically after a 24-h incubation, hypoxanthine was found in amounts roughly equivalent to the decrease in adenine concentration. The desamination product was separated from the starting material by the method of WEISSMANN, BROMBERG AND GUTMAN[14]. After extraction from the paper, hypoxanthine was identified by measuring its λ_{max} at 3 different pH values (see Table III).

TABLE III

CONVERSION OF ADENINE INTO HYPOXANTHINE BY *Pseudomonas aeruginosa* BDH

A bacterial suspension of $5 \cdot 10^9$ cells was incubated with 100 µg/ml adenine. Temperature 30°, pH 7.5. After 24 h, the mixture was centrifuged and the supernatant subjected to chromatography, using the solvent: *n*-butanol–1 % ammonia (6:1) v/v[14]. The spots were then extracted and their spectrum measured at 3 different pH values.

Substance	R_F	*Absorption maximum (mμ) at pH*		
		5.5	*10.5*	*12.7*
Adenine	0.55	259.5	256	256
Hypoxanthine	0.47	251	260	263
Product, obtained from chromatogram	0.46	251	260	262

TABLE IV

OXIDATION OF 2-AMINOPURINE TO 2-AMINO-8-HYDROXYPURINE BY *Pseudomonas aeruginosa* BDH

Experimental conditions as in Table III. Solvent used for chromatography: 95 % ethanol–water–glacial acetic acid (85:10:5) v/v (solvent A); 95 % ethanol–12.5 % ammonia (80:20) v/v (solvent B).

Substance	*R_F in solvent*		*Absorption maximum (mμ) at pH*	
	A	*B*	*5.5*	*8.0*
2-Aminopurine	0.63	0.62	304	304
2-Amino-8-hydroxypurine	0.51	0.39	305	305
Product, obtained from chromatogram	0.50	0.36	305	305
Guanine	0.46	0.37	246 275	246 275

2-Aminopurine was slowly oxidised to its 8-hydroxy derivative. The latter accumulated in the medium, as it is resistant to attack by the bacterial enzyme, in sharp contrast to its behavior towards mammalian XO[3]. The oxidation product was identified by its R_F value and by the fact that its absorption spectrum is close to that of the starting material[3] (see Table IV). No trace of the alternative oxidation product guanine was found.

Methylated adenines as substrates of mammalian XO

6-Methylaminopurine (IV), like adenine[1] and kinetin[15], is attacked first at position 8. This is demonstrated in Fig. 4, where a peak at 273 mµ appears in the intermediary stages of the enzymic reaction and vanishes again towards the end of the oxidation. If the corresponding isoguanine derivative would have been formed, its presence would have been noted by a temporary peak at about 285 mμ[1]. The results of Fig. 4 are therefore completely analogous to those with kinetin and establish the main course of oxidation, although they do not exclude the possibility that the alternative pathway may be used to a very small extent. When the reaction of (IV) with XO was stopped at an intermediary stage, chromatography indicated the presence of 3 components, corresponding to (IV), a single intermediate (V) and the end-product, 2,8-dihydroxy-6-methylaminopurine (VI) (see Table V). The change of O.D. at 284 mμ, the isosbestic point of (IV) and (VI), is plotted in Fig. 5 as function

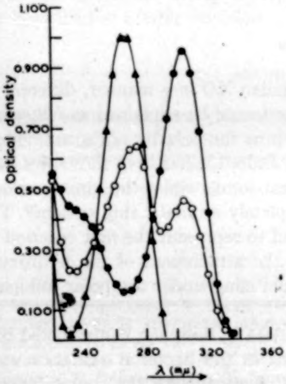


Fig. 4. Spectral changes during the enzymic oxidation of 6-methylaminopurine (IV). Substrate: $6.7 \cdot 10^{-5}$ *M*; other conditions as in Fig. 2. ▲—▲ Zero time: Spectrum of (IV). ○—○ After 35 min: Two new peaks have appeared, at 273 mμ (V) and 302 mμ (VI), respectively. ●—● After 90 min: Reaction completed; spectrum of VI.

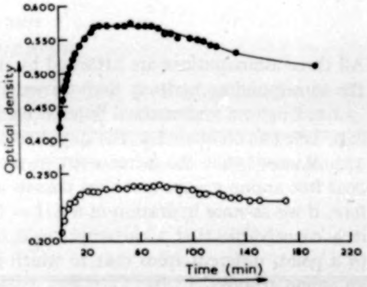


Fig. 5. Spectroscopic evidence for the formation of an intermediate, bearing an 8-hydroxyl group, during the enzymic oxidation of 6-methylaminopurine (●—●) and adenine (○—○). The changes in O.D. at the isosbestic points of 6-methylaminopurine (IV) and its 2,8-dihydroxy derivative (VI) (284 mμ) and of adenine and its final oxidation product (277 mμ) are plotted as function of time. XO, 1:200; catalase, 1:500. Temperature 28°; pH 8.0. Substrates: 6-Methylaminopurine, $6.7 \cdot 10^{-5}$ *M*; adenine, $4.7 \cdot 10^{-5}$ *M*.

TABLE V

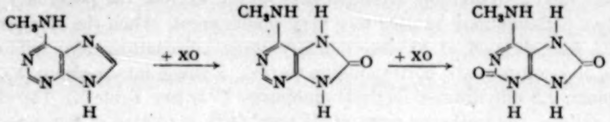
PATHWAY OF OXIDATION OF 6-METHYLAMINOPURINE BY MAMMALIAN XANTHINE OXIDASE

Substrate, $1.3 \cdot 10^{-4}$ *M*; XO, 1:200; catalase, 1:500; 10^{-3} *M* phosphate buffer, pH 8.0. Temperature 28°. The reaction was stopped after 55 and 200 min respectively by immersion into boiling water for 5 min. The paper chromatograms were developed with solvent A (see Table IV). Since the reaction products did not fluoresce on paper, each strip was cut into 10 sections, parallel to the starting line; each section was extracted with water and the u.v. spectrum of all extracts measured. Therefore the R_F values are only approximate.

Substance	R_F	*Absorption maximum (mμ) at pH*		
		5.0	*8.0*	*10.0*
Starting material (IV)	0.8	267	267	269
Intermediate (V)	0.7	272	273	281
End-product (VI)	0.3	304	302	300

of time, for comparison with the analogous curve for adenine. The latter is shown here again, in order to correct a technical error in Fig. 2 of our previous paper[15].

6-Dimethylaminopurine, like 7- or 9-methyladenine, was found refractory to XO. Similar observations have been made previously by Dr. G. B. ELION[16].



(IV) (V) (VI)

DISCUSSION

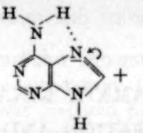
All three aminopurines are attacked by mammalian XO in a manner, different from the corresponding hydroxy derivatives. This fact could be explained as follows:

1. Due to a systematical difference in structure, the polarity of the aminopurines is reversed as compared to the oxy derivatives. Indeed it has been shown by BROWN AND MASON[17] that the latter exist in the lactam form, whilst the aminopurines all bear free amino groups and thus possess a completely aromatic ring system[18]. Therefore, if we assume hydration of a CH = N bond to represent the first reaction step, it is conceivable that a carbonyl could direct the attachment of the hydroxyl ion to a point, different from that to which it would cling under the polar influence of an amino group.

2. This interpretation of the experimental data is, however, contradicted by the observation that the pathway of 2-aminopurine in the bacterial oxidation system differs from that with mammalian XO, but is identical with the course, taken by the oxidation of 2-hydroxypurine with the latter enzyme. Clearly, it is not so much the intrinsic polarity of the substrate as the polarity induced by complex formation with the enzyme, that determines the point of attack. Aminopurines, *e.g.*, could combine with XO by attaching their amino groups to a negatively charged phosphate group, present in the flavin-adenine-dinucleotide[11], or by forming a hydrogen bridge, *e.g.* with a carbonyl group in the active center.

The assumption that the specific mode of attachment of a substrate to the active surface determines the pathway of oxidation is also supported by earlier observations revealing characteristic differences between mammalian and bacterial enzyme. Thus, 7-methylhypoxanthine is refractory towards the former, but is oxidised by the latter to 7-methylxanthine[5]. Similarly, 3-methylxanthine is converted only by the bacterial enzyme to 3-methyluric acid.

Adenine is oxidised by XO only if specific structural requirements are fulfilled: (a) At least one hydrogen atom must be present in the 6-amino group. (b) The imidazole ring must contain a free NH group. The resistance of 6-dimethylaminopurine to enzymic attack may be ascribed to steric hindrance, but such an explanation appears unsatisfactory in view of the fact that the large furfuryl group of kinetin does not prevent reaction at C-8. More probably, requirement (a) indicates hydrogen bonding either to an acceptor group in the enzyme surface or to N-7 in the purine system (see MASON[18]):



In this representation of the adenine structure, polarisation of the $\overset{8}{C} = \overset{7}{N}$ double bond would make C-8 susceptible to attack by an hydroxyl ion. A choice between the two modes of hydrogen bonding—whether inter- or intramolecularly—could be made by introducing mono- and dimethylamino substituents into positions 2 or 8, where an intramolecular bridge is no longer possible. Experiments in this direction will be described at a later occasion.

ACKNOWLEDGEMENTS

The authors wish to express their sincere thanks to Prof. F. Bergel and Dr. R. C. Bray for the supply of purified milk xanthine oxidase and to Drs. R. K. Robins, A. Bendich, G. B. Elion and E. C. Taylor for the generous gift of purine derivatives. They are also obliged to Mr. M. Chaimovitz and R. Knafo for their expert technical help.

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A HIGHLY ACTIVE CARBAMATE KINASE AND ITS APPLICATION TO THE REGENERATION AND PREPARATION OF [^{32}P]ADENINE NUCLEOTIDES

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(Received April 15th, 1959)

SUMMARY

A procedure for the preparation of highly active carbamate kinase from streptococci has been presented. The purified preparation at 38° and pH 7.4 catalyzes the transfer of 780 μmoles of phosphate per mg per min from carbamyl phosphate to adenosine diphosphate.

The usefulness of the purified enzyme for adenosine triphosphate regeneration and for ^{32}P labelling of adenine nucleotides has been discussed. As examples, the preparation and use (completed in 1 h in some cases) of the following compounds have been described: ATP labelled solely in the γ phosphate, in the β position, or equally in β and γ, and ADP labelled in the β position.

INTRODUCTION

Carbamate kinase was recognized first by JONES *et al.*[1], who also purified partially the enzyme from streptococcus fecalis[2] and indicated the reversibility of the reaction according to equation (1).

$$CO_2 + NH_3 + ATP^* \rightleftharpoons \text{carbamyl phosphate} + ADP \qquad (1)$$

We noticed[3–5] that sonicated preparations from Strain D_{10} Group D streptococci have a very high activity for reaction (1). We have purified the enzyme from this source and have used it as a convenient ATP regenerating system from carbamyl phosphate, since the equilibrium of reaction (1) is far towards ATP synthesis[2]. This fact and the availability of the purified enzyme permits, in less than one hour's work and in the most simple manner, the labelling of ATP with ^{32}P. The purification of the streptococcal carbamate kinase, and examples of its application in labelling adenine nucleotides is presented in this paper.

Abbreviations used in this paper are as follows: CP, carbamyl phosphate; AMP, 5′-adenylic acid; ADP, ATP, adenosine di- and tri-phosphate; G6P, glucose-6-phosphate; ARP^{32}P, β labelled ADP; ARP^{32}PP, ARPP^{32}P and ARP^{32}P^{32}P, β-labelled, γ-labelled, and β,γ-labelled ATP; Pi, inorganic phosphate; EDTA, ethylenedinitrilotetraacetate; Tris, Tris-hydroxymethyl methylammonium; 1,3 DPGA, D-1,3-diphosphoglyceric acid.

MATERIALS AND METHODS

AMP and ADP were obtained from Pabst Laboratories, Inc., KCNO was the J. T. Baker reagent. $Na_2H^{32}PO_4$ was obtained from Dr. C. Huggins, Department of Pharmacology. Adenylate kinase was prepared by the method of Colowick and Kalckar[6] and hexokinase, fraction 3a, by the method of Berger *et al.*[7].

The adenine nucleotides, glucose, G6P and Pi were separated on Whatman No. 2 paper by ascending irrigation with a solvent composed of 8 volumes 2-propanol plus 10 volumes of a mixture composed of 51 volumes 99 % acetic acid, 50 volumes 1 *M* NH_4OH, and 1.5 volumes 0.1 *M* Na_3EDTA. The adenine spots were located with u.v. light and the phosphate esters were located by the procedure of Wade and Morgan[8] as modified by Runeckles and Krotov[9]. Glucose was detected by the aniline–phthalic acid spray[10]. R_F's obtained were as follows: ATP, 0.10 ± 0.01; ADP, 0.20 ± 0.00; AMP, 0.40 ± 0.00; Pi, 0.56 ± 0.02; glucose, 0.54 ± 0.02; G6P, 0.29 ± 0.00.

EXPERIMENTAL

Purification of carbamate kinase

All operations during fractionation were carried out at 0° and all centrifugations were at 4,000 × *g* unless otherwise indicated. The ammonium sulfate solutions were at pH 7.4 and saturated at 0°. Strain D_{10}, Group D streptococci was grown according to the optimum culture medium of Slade and Slamp[11] except that Casitone was replaced by Tripticase BBL. After each 17-h growth at 38° the culture was used to inoculate 10 times the volume of a fresh medium. Normally 4 to 5 passages were carried out. The cells were harvested by centrifugation, washed twice with distilled water, suspended in an amount of water approx. 1/40 the volume of the medium used and sonicated for 25 min at 0° in a Raytheon Oscillator Magnetostrictor. The preparation was then centrifuged for 30 min and the supernatant fluid was then recentrifuged at 20,000 × *g* for 20 min. The supernatant fluid was then lyophylized. The lyophilyzed preparation was taken in 0.04 *M* $NaHCO_3$ to a concentration of 10 mg protein/ml. In a typical preparation 60 ml of the solution (Crude Fraction, see Table I) were mixed with 60 ml of a Bentonite suspension (washed as previously described[12]), containing 18 mg Bentonite/ml. After 15 min of contact with occasional stirring the mixture was centrifuged for 15 min, the precipitate discarded and the

TABLE I

PURIFICATION OF CARBAMATE KINASE

Although the purification procedure is very reproducible for a certain batch of lyzed bacteria, some variations have been encountered during purification from batch to batch. Preliminary titration particularly for the bentonite treatment is recommended.

Fraction	*Volume ml*	*Total protein mg*	*Total units (thousands)*	*Specific activity*	*Yield %*
Crude	60	600	297.6	496	100
Fraction I	103	226	255.4	1130	86
Fraction II	25	143	220	1538	74
Fraction III	16	36	134.5	3700	45.2
Fraction IV	6	12	105	8750	35.4

supernatant fluid (Fraction I, see Table I) was mixed with 2 volumes of acetone at —10° and then centrifuged at this temperature for 15 min. The precipitate was taken up in 25 ml of water and any insoluble material discarded after centrifugation. The supernatant fluid (Fraction II, see Table I) was mixed with 36 ml of ammonium sulfate and the precipitate discarded. The supernatant fluid was mixed with 36 ml of ammonium sulfate and the precipitate collected and dissolved in water to a volume of 16 ml (Fraction III, see Table I). Fraction III was then mixed with 15 ml of acetone at —10° for 15 min. The supernatant fluid was collected and mixed with 15 ml of acetone at —10° and the precipitate collected and dissolved in water to give Fraction IV (see Table I).

Assay for carbamate kinase

The following components were mixed in a final volume of 1.0 ml; 100 μmoles of Tris buffer pH 7.4; 2 μmoles of ATP; 20 μmoles of carbamyl phosphate and 5 μmoles of Mg^{++}, 50 units of hexokinase and 90 μmoles of glucose. After 10-min incubation 1.0 ml of 0.5 *N* HCl was added and the mixture heated for 2 min at 100°. Aliquots of the supernatant were assayed for inorganic phosphate by the method of GOMORI[13]. The disappearance in easily hydrolyzable phosphate should correspond to the amount of glucose-6-phosphate formed and therefore equal the transfer of phosphate from CP to ADP. Under the above conditions there is proportionality for enzyme activity up to 15 units. Control tubes without carbamate kinase are carried out to estimate by difference the amount of transfer. Although CP preparations are always contaminated with Pi in addition to the small amounts of Pi formed by spontaneous decomposition of CP and from ATP during the hydrolysis, this does not interfere with the assay; since, as described above, control tubes are used.

Definition of enzyme units and specific activity

A unit is defined as the amount of enzyme which catalyzes the transfer at pH 7.4 of 1 μmole of phosphate from carbamyl phosphate to ADP; the transfer of the terminal phosphate of ATP to glucose, and the transfer of the terminal phosphate of ATP to AMP all at 38° and for 10 min. Specific activity is defined as the enzyme units per mg protein.

Preparation of ARPP^{32}P

10 μl 1 *M* $Na_2H^{32}PO_4$ (specific activity about 0.2 C/mole), 10 μl 1 *M* sodium acetate, pH 4.92 and 25 μl 1 *M* KCNO (prepared immediately before use) were mixed and incubated at 30° for 15 min. An additional 25 μl KCNO was added and the mixture incubated 15 min more, following which the tube was chilled in ice. To the cold mixture was added 0.20 ml 40 m*M* K_2ADP, 0.05 ml 1 *M* Tris-Cl, pH 7.30, 0.01 ml 1 *M* Mg SO_4 and 20 units of Fraction IV of carbamate kinase in 0.1 ml. This mixture was incubated 30 min at 38° and the reaction was stopped by heating the mixture at 100° for 1 min.

To confirm the position of ^{32}P in the ATP, the separation on paper was performed as outlined above and the ATP was eluted. To the ATP eluate (1 ml, 2 μmoles) were added 0.01 ml 1 *M* Mg Cl_2, 5 μl 1 *M* glucose, 0.02 ml 1 *M* Tris-Cl, pH 8.5, and 0.05 ml hexokinase (30 units). The mixture was incubated 20 min at 38° and was stopped by heating 1 min at 100°. Part of this mixture was applied to Whatman No. 2 paper

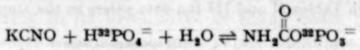
TABLE II

SYNTHESIS OF ARPP^{32}P

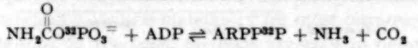
The radioactivity values represent those of the applied aliquot of the deproteinized mixture after the formation of AT^{32}P and its use in the hexokinase system (see text). Occasionally, due to a trailing of Pi, small amounts of radioactivity appeared in the AMP and ADP areas.

Component	Net radioactivity in component	
	After synthesis	After hexokinase action
	(counts/min × 10^{-4})	
Pi	14.0	
AMP	0.0	
ADP	0.0	0.0
ATP	16.2	
G6P		16.0

and was separated as described above. The results given in Table II show that the radioactivity is distributed as predicted from eqns. (2), (3) and (4):



$$KCNO + H^{32}PO_4^{=} + H_2O \rightleftharpoons NH_2\overset{O}{\overset{\|}{C}}O^{32}PO_3^{=} \qquad (2)$$



$$NH_2\overset{O}{\overset{\|}{C}}O^{32}PO_3^{=} + ADP \rightleftharpoons ARPP^{32}P + NH_3 + CO_2 \qquad (3)$$

$$ARPP^{32}P + glucose \rightleftharpoons G6^{32}P + ADP \qquad (4)$$

Preparation of ARP^{32}P and ARP^{32}PP

The procedure given here illustrates permissible variations in technic. C^{32}P was prepared as described above, on a double scale, and 0.10 ml 40 m*M* K$_2$ADP, 1.0 ml 40 m*M* K$_2$AMP, 20 units of carbamate kinase (Fraction IV) in 0.1 ml 1 *M* Tris-Cl pH 7.3, 1 *M* 0.02 ml MgSO$_4$ and 0.1 ml myokinase (25 units) were added. This mixture was incubated 30 min at 38° and was stopped by the addition of 0.1 ml 1 *M* TCA. The precipitated proteins were centrifuged off and the supernatant was extracted 3 times with 5 volumes of diethyl ether. This TCA-free solution contains the ARP^{32}P.

ARP^{32}P isolated from half of the preparation just described was added to CP (prepared from K$_2$HPO$_4$), plus the other components of the carbamate kinase reaction as indicated before. In this case isolation of the ARP^{32}P was obviously necessary to ensure freedom from ^{32}Pi and adenylate kinase. The reaction was allowed to proceed 20 min at 38° and was stopped by 0.1 volume of 1 *M* TCA. The protein-free supernatant was extracted as before with diethyl ether. This TCA-free solution contains the ARP^{32}PP.

To confirm the position of the ^{32}P, an aliquot containing 2 μmoles AT^{32}P was treated with the hexokinase system as before. A sample of the product of the reaction was applied to paper and developed. The results of these experiments are in Table III. The data show that the combination of adenylate kinase and an excess of AMP yielded preferentially ARP^{32}P. It is also seen that the non-radioactive Pi introduced into the AD^{32}P to form AT^{32}P was transferred to G6P which was ^{32}P-free. Therefore, the AT^{32}P was ARP^{32}PP.

Preparation of ARP^{32}P^{32}P

The procedure used in this case is identical to that used in preparing ARP^{32}P

TABLE III

SYNTHESIS OF ARP^{32}P AND ARP^{32}PP AND THE HEXOKINASE REACTION WITH ARP^{32}PP

The values represent those of the aliquot used.

Component	*Net radioactivity in component*		
	Synthesis of AD^{32}P	*Synthesis of AT^{32}P*	*Hexokinase action*
	(counts/min × 10^{-3})		
Pi	14.0	0.0	
AMP	0.0		
ADP	15.2	2.2	5·3
ATP	1.0	3·8	
G6P			0.0

TABLE IV

PREPARATION OF ARP^{32}P^{32}P AND THE HEXOKINASE REACTION

As indicated in Tables II and III the data refers to the aliquots analyzed.

Component	*Net radioactivity after*	
	Synthesis	*Hexokinase action on AT^{32}P*
	(counts/min × 10^{-3})	
Pi	3·8	
AMP		
ADP	2.24	4·62
ATP	3·75	0·51
G6P		4·80

except that no AMP was added. The ATP formed by the carbamate kinase is completely equilibrated with that formed by the adenylate kinase. Thus the β and γ positions are equally labelled. The isolated AT^{32}P was then tested with hexokinase and the results are expressed in Table IV.

The data illustrate that equal amounts of ^{32}P are found in the G6P and in the ADP formed from the hexokinase reaction. Isolation of the AT^{32}P was chosen to simplify the results of the hexokinase reaction, but under other circumstances isolation may have been neglected.

DISCUSSION

The purification of carbamate kinase presented here yields preparations of a very high specific activity which are stable for several months at —20°.

The purified preparations serve as a convenient specific regenerating system for ATP from ADP and with crude preparations or in the presence of adenylate kinase for the generation of ATP from AMP and catalytic amounts of ATP or ADP; since the equilibrium of reaction (1) is in favor of ATP synthesis. In addition since the preparations are free of ATP-ase and CP is the phosphate donor, ATP-ase like action due to 1,3 DPGA decomposition[14] is not a problem as when using phosphoglycerates for ATP regeneration. However, CP is also decomposed in most tissues enzymically by acyl phosphatase[15, 16] and although not as unstable as 1,3 DPGA, has a very short half life. The main drawback of the system for ATP regeneration is the fact that NH_3 and CO_2 are liberated in addition to inorganic phosphate, which may be a disadvantage in certain studies.

The available methods for the preparation of ^{32}P adenine nucleotides based on the use of crude biological materials[17–19] suffer from disadvantages of low yield, low specific activity or random labelling; somewhat better yields have been obtained with purified enzymes[20]. A more recent method[21] involving the use of dicyclohexylcarbodiimide avoids biological preparations but leads to laborious procedures, and the derived materials are contaminated by a variety of side products.

The procedures described here permit the preparation, with minimal effort, of adenine nucleotides of specific activity equal to the specific activity of the ^{32}P added and in yields of 50 % or more (based on ^{32}P). In some cases isolation of the products may be desired to remove starting materials. The products are labelled specifically and interfering side products do not appear in measurable quantities.

In the method described here C^{32}P is generated *in situ*, isolation of which is, in fact, undesirable owing to its instability. In many cases the enzymic products could be used subsequently without isolation. When adenylate kinase is used during the preparation, separation would be mandatory for most purposes.

Additional variations on the basic methods are easy to design. To label other nucleotides, it would be a simple matter to include the appropriate precursor, *e.g.* uridine diphosphate and nucleotide diphosphokinase.

ACKNOWLEDGEMENTS

This work was aided by grants from the Helen Hay Whitney Foundation and the National Institutes of Health (A-1855-C4) and The Kansas Heart Association. We are indebted to Dr. R. BROWN for help in some of the work reported here.

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THE OSMOTIC PUMP THEORY OF SELECTIVE TRANSPORT

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(Received April 11th, 1959)

SUMMARY

A theory of the nature and functioning of the cytoplasmic membrane has been proposed. It is based on the concept that the main barrier to diffusion into and out of the cell is a dense gellular layer. Enzyme action is considered to increase and decrease the number of charged groups within this layer in such a way that the resulting osmotic pressure changes bring about a reciprocating uptake and expulsion of solution by the membrane itself. An attempt is made here to show that presupposing the existence of this pumping motion and of the presence of specific complexing groups attached to the gel matrix, it is possible to obtain a consistent explanation for a number of phenomena exhibited by the cytoplasmic membrane, such as passive, facilitated and active transport; osmotic lysis; "flickering" of red blood cells; and transmission of nervous impulses.

INTRODUCTION

The term *selective transport* refers to the mechanism whereby living cells absorb or excrete specific chemical compounds while remaining indifferent to other, often closely related, chemical species. Transport which operates on a *specific* compound in such a direction as to make the concentration of a compound in the cell equal to the external concentration is termed *facilitated*[1], while movement which tends to set up a concentration gradient between the internal and external solutions is referred to as *active* transport.

Selective transport has many characteristics in common with enzyme mechanisms, especially in regard to chemical specificity[2], kinetics[3], inhibition[3], competition[4], species differences[5], and finally the ability to be induced in bacteria[6]. These characteristics suggest the formation of complexes between the transported (or substrate) molecule and a component of the cytoplasmic membrane (variously referred to as transport site, carrier molecule or permease) in much the same way as complex formation in enzyme processes. How such complexes result in transport, however, is the essential problem still facing workers in this field. One possible explanation forms the basis of the present paper.

Outline of the osmotic pump theory

This section will consist of a brief outline of the proposed mechanism, and will be followed by evidence offered in support of the main premises of the theory.

The diffusion barrier of the cell is considered to be a dense gellular layer of the order of a few hundred Ångstrøms in thickness, which together with an overlying lipid layer constitutes the cytoplasmic membrane. This gel contains a number of lipoproteins and proteins with lipophilic side chains which would confer on the gel a somewhat hydrophobic nature, so that even when completely hydrated the water content is low (probably of the order of 50 %). Such a dense gel will have only very small interstices and it is postulated normally will allow only the passage of molecules as small as water. The outer lipid layer is not considered to be a barrier to diffusion but is responsible for the surface characteristics of the cell.

It is suggested further that enzymes are present in the gel and probably help to make up the gel matrix. Certain of these enzymes, such as esterases and anhydrases, are considered to be activated periodically and to produce a number of osmotically active groups within the gel. This increases the hydrophilic nature of the gel and its internal osmotic pressure rises above that of the ambient solution, with the result that water both from inside and outside the cell flows into the membrane causing it to swell.

The existence of the osmotically active groups is considered to be transitory however, their production lasting only a short time, after which they decay either spontaneously or as a result of further enzyme action. The internal osmotic pressure of the gel then drops below that of the ambient solution, and a flow of water out of the membrane results. Continued repetition of this sequence of events produces a reciprocating movement of water into and out of the membrane gel, and it is this pumping mechanism brought about by processes linked directly with the metabolism of the cell which is considered by the hypothesis to be the main driving force for the transport of materials through the cytoplasmic membrane.

Having established a means by which materials may be moved into and out of the gel let us next see how this mechanism could be made to transport specific molecules through the gel. During the initial stage of the uptake phase of the pumping cycle, only small molecules such as water can enter the gel matrix due to the small size of the interstices. However, as swelling proceeds, the interstices increase in size and a point is reached at which a given molecule can enter the gel, and does so, being swept inward by the flow of water. The distance to which a molecule penetrates the gel will be limited since the inward flow of water soon ceases and the molecule is carried out again by the subsequent outward flow. Diffusion within the gel matrix will be very small (for reasons presented later) so that the membrane as pictured so far will be virtually impermeable to all molecules other than water.

However, let us go one step further and postulate the existence within the gel matrix, of a number of complexing sites *C*, attached directly to the gel matrix and capable of forming a relatively loose complex *X* with a specific substrate *S* in a reversible manner, thus:

$$S + C \rightleftharpoons X \tag{1}$$

The entrance of *S* into such a gel will bring about the formation of complexes, the number of which is dependent on the concentration of *S*. Any molecules of substrate not complexed will be swept out by the next contraction phase. At the same time a certain number of complexes will decompose during the contraction phase, but provided the rate of decomposition of *X* is not too large by comparison with the rate of efflux, a number of substrate molecules will remain complexed until after their free movement has been restricted by the contracting matrix. A fraction of these complexes will then decompose to build up the substrate concentration within the gel to a new equilibrium value. These newly released molecules thus are available to be swept further into the membrane gel on the next pumping cycle, and by coming into contact with more complexing sites they can continue to move further through the gel with each cycle. In this way the movement of S through the membrane gel has been "facilitated" by the presence of complexing groups. This facilitation is equal in both directions through the membrane, so that net movement of material always occurs from a region of higher toward a region of lower electrochemical potential. This, then is an example of facilitated transport only.

It is probable that the properties of many chemical groupings on the matrix of such a gel would be altered during changes from a hydrophobic to a hydrophilic gel. These changes could conceivably effect some complexing groups in such a way that their ability to complex during one half the cycle is greater than during the other. Such "differential" sites present on the walls of a channel within the gel would have the following effect. When the water moves past them in one direction little or no complexing occurs and the substrate is carried along with the water. When the direction of flow reverses however, the sites are able to complex the substrate and prevent its movement. Under these conditions movement is facilitated in one direction more than another, allowing the substrate to move against an electrochemical potential gradient and resulting in *active* transport.

Having established the essential mechanism of the theory, let us next consider some of the assumptions on which it is based, and in so doing elaborate on the above model.

Evidence of the nature of the cytoplasmic membrane

Chemical composition: Most information in this field has been obtained from work done on the ghosts of erythocytes[7–9], and of the protoplasts of bacteria[10, 11] prepared by osmotic lysis. Such ghosts were found to consist of both lipids and proteins, the ratio varying from 2–5 times protein over lipid depending on the method of preparation. A varying amount of carbohydrate was also found in bacterial ghosts but this fraction may have originated during digestion of the cell walls.

Physical nature: Again we must turn to the red cell ghost for information on the physical nature of the membrane since most work has been done on this preparation.

A number of estimates of the thickness of the dehydrated ghosts have been made, and depending on the method of preparation values ranging from 50 to 1000 Å have resulted[9]. Most, however, are the range 100–300 Å. The only direct estimate of the amount of water present in the ghosts in their hydrated condition is that of Ponder[12] who puts it at 67 % indicating a thickness of 300–900 Å. in the living cell.

Evidence concerning the surface ultrastructure of red blood cells has been obtained from the application of the electron microscope. (For a review of this work see Ponder[8].) One model of the structure of ghosts arising from all this work which seems to have reasonable support, however, is that of a fibrous structure on the surface of which is a layer of plaques or cylinders about 30 Å high and 200 Å in diameter held together by ether-extractable lipids[13].

Frey-Wyssling[14] has developed the idea that not only the cytoplasmic membrane but the entire cytoplasm as well possesses a molecular organization consisting of a network of both globular and filiform proteins stretching throughout the aqueous phase and held together by points of attachment or junctions between molecules. The viscosity of this structure will depend on the nature of the forces operating at the junctions and the amount of protein matrix present. In the interior of the cell, the matrix is thin and the junctions weak resulting in a thixotropic structure which will flow under relatively little stress. Toward the surface of the cell, however, the matrix becomes more dense and the junctions stronger resulting in a tough but elastic gel on the outer surface of which will be adsorbed a layer of lipid molecules.

An important characteristic of gels is their ability to swell or shrink in accordance with their affinity for the medium in which they are immersed. Gels which are lipophilic in nature would be expected to repel the water and to shrink in an aqueous medium, while the presence of ions within a gel will cause the entrance of water due to osmotic pressure, and expansion of the structure of the gel. The extent of swelling will depend on the nature of the bonds holding the fibers or spheres together. If there is a large percentage of chemical bonding, swelling will be slight whereas if bonding is by cohesion only, swelling will be extensive and might even result in a complete dispersion of the matrix.

In the present theory, the essential ideas of FREY-WYSSLING are retained. In most cells the cytoplasm is pictured as consisting of a proteinaceous gel which increases in density and becomes more fibrous toward the cell surface. With this increase in density of protein, there is also an increase in lipid content, and in the lipophilic side chains attached to the proteins, so that the gel tends to be more hydrophobic, less hydrated and more compact toward the outer surface of the cell. On the outside of this gel is a layer consisting mainly of adsorbed lipids or possibly having the "plaque" structure mentioned above, which gives rise to the "membrane" observed in some electron micrographs. This thin film is not likely to be capable of all the dynamic changes which the permeability barrier must undergo, and so it is not considered to be important in determining the permeability characteristics of the surface structure, rather it is considered to have a porous nature, and to be freely permeable to all but the largest molecules. This again is consistant with the "plaque" structure since the spaces between the close-packed cylinders could act as pores.

A further consideration of importance to the present discussion is the state of the water in the gel interior. According to the concepts of DE BOER AND ZWIKKER[15] and of BRADLEY[16], the adsorption of water on solids results in the formation of a primary layer which, due to the dipole nature of water, induces the formation of further layers, so that in a gel a very high degree of order will exist throughout the water phase. BRADLEY'S equations, based on this concept, have been tested by HOOVER AND MELLON[17] on data obtained from the measurement of water adsorption by a number of proteins with excellent agreement over a wide range. This concept is confirmed further by thermodynamic measurements, which indicate that during the initial uptake of water by proteins there is a very large decrease in entropy[18].

The effect of polar forces on water molecules can be illustrated also by consideration of the hydration of ions. Two types of hydration layers surround an ion in solution. The inner and more strongly bound layer has been termed the primary sheath by BOCKRIS[19]. It consists of a discrete number of water molecules held so firmly by electrostatic forces that it moves as an entity with the ion. The secondary sheath is more diffuse but involves molecules several molecular diameters from the ion. With reference to the present discussion the most important features of the hydration sheath are firstly that the water molecules, being dipoles, are highly oriented and as indicated by the greater density of this layer, are therefore, more tightly packed than those in the bulk of the solution, and secondly that molecules at a given distance from the ion but within the sheath are capable of translational motion in *2 directions only, motion toward or away from the ion being highly restricted.*

Returning now to our membrane gel, it is obvious that nearly all the water will be in a sort of semicrystalline state in all channels whose radii are of the same length as 2 or 3 water molecules, *i.e.* having diameters of the order of 10 Å or less. Though highly restricted in their radial motion within such channels, the water molecules are still capable of normal motion parallel to the walls of the channel so that mass flow through the gel is virtually unrestricted. Any solute molecules within the channels, however, will experience considerable resistance to diffusion since for this to occur, water molecules must be moved from in front and deposited behind them, and this can hardly be done without radial movement.

We may distinguish then 3 sizes of channels or interstices in the cell: (a) Those large enough to admit water molecules but too small for most solute molecules, *i.e.* those having maximum diameter of 2–3 Å. (b) Those large enough to allow the entry of solute molecules but small enough that surface forces prevent the motion of these molecules independently of the water medium (these are probably of the order of 10 Å). (c) Those large enough to allow virtually normal diffusion of solute molecules, *i.e.* those of a higher order than 10 Å.

Under normal conditions the gel is considered to possess channels of size (a) in its contracted form and size (b) in its expanded form. When subjected to a solution of very low tonicity, however, the membrane gel expands beyond its normal limit forming channels of size (c) which allow free passage of nearly all compounds.

This latter condition, the existence of large pores, occurs during osmotic lysis and provides a valuable clue to the structure of the cytoplasmic membrane. An extensive study of osmotic hemolysis of red blood cells has shown that the release of hemoglobin is not the result of rupturing of the cytoplasmic membrane but rather is an event which occurs evenly over the entire cell surface. The cell then recovers rapidly, preventing any further loss[20], and exhibiting permeability characteristics similar to the original cells[21,22]. What appears to happen is that as the external solution becomes less tonic the cell, acting as as osmometer increases its volume until a point is reached at which the membrane suddenly becomes porous, even to hemoglobin molecules (diameter about 75 Å[14]). Under these conditions the maintenance of the osmotic pressure is no longer possible and the cell contents are ejaculated as the cell returns to its normal shape[23].

The change in porosity of the membrane with decreasing tonicity is consistent with the concept of the osmotic barrier as being a gel, since the swelling of such a structure would be limited by the presence of a number of junctions up to the point at which the internal osmotic pressure exceeds the forces operating at the junctions. When this occurs there would be a sudden increase in pore size bringing about the osmolytic condition.

Kinetic treatment of the osmotic pump mechanism

The kinetic aspects of selective transport are fairly well known, and for any theory to be acceptable it should lead to the derivation of equations describing, at least qualitatively, the general nature of these observations. Success in this does not prove a theory since a number of models can lead to the same equations; however, failure to do so obviously makes a model suspect.

Before applying this test to the present theory let us consider the pumping mechanism in more detail. The production of osmotically active species results in the expansion of the membrane and in effect causes the gel matrix to expand outward through the inner and outer solutions. This is best illustrated in Fig. 1 in which are plotted as a function of time the distance from the center of the membrane (*O*) of a number of points fixed to the gel matrix. Those points located in the outer half (outer pumping zone) of the membrane move through the outer solution while those in the inner pumping zone move into the cytoplasmic solution.

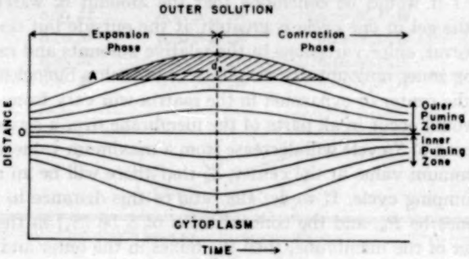

Fig. 1.

Facilitated transport: Consider a solute having a concentration $[S_0]$ in the outer solution and $[S_m]$ ar a depth d_s in the expanded membrane, for which complexing sites exist throughout the gel matrix at a concentration N_p. When the membrane is fully contracted, an equilibrium as in eqn. (1) results for which the constant is

$$K = \frac{[S_m]\,[C]}{[X_m]} \tag{2}$$

where $[C]$ is the concentration of free sites. Further, since $[C] = N_p - [X_m]$, then

$$[X_m] = \frac{N_p\,[S_m]}{K + [S_m]} \tag{3}$$

As the gel expands and the interstices increase in size a point is reached (*A*, Fig. 1) at which *S* starts to enter the gel following which it penetrates to the depth and for the time indicated by the shaded area in Fig. 1. In this region the substrate concentration is considered to be the same as in the outer solution so that a new equilibrium is reached in which the concentration of complexes becomes

$$[X_0] = \frac{N_p[S_0]}{K + [S_0]} \tag{4}$$

If now for the sake of simplicity we consider that none of these complexes decomposes until contraction is completed, then the net amount of S entering the gel will be the difference between the amount of S carried outward as a complex during expansion and the amount carried inward as a complex during contraction. The inward flux of S can be defined then as

$$\begin{aligned} F &= ([X_0] - [X_m])d_s W \\ &= \left(\frac{N_p\,[S_0]}{K + [S_0]} - \frac{N_p\,[S_m]}{K + [S_m]}\right) d_s W \end{aligned} \tag{5}$$

where d_s is now the distance penetrated by S in one cycle and W is the frequency of the pump. Note that if $[S_m] > [S_0]$, F is negative indicating an outward movement of S. The term $([X_s] - [X_m])/d_s$ will be the concentration gradient near the outer surface of the gel. It can easily be shown that whenever such a gradient exists at any point in the gel at which the pumping motion is in operation there will be a net flow of material past this point toward the region of lower concentration.

From Fig. 1 it would be concluded that the amount of water flowing past a given point in the gel in one cycle is greatest at the outside but decrease to zero at the center. However, since variations in the relative amounts and rates of expansion of the 2 pumping zones undoubtedly would occur in such a biological system as this, the position of the center of expansion in the matrix will vary from cycle to cycle so that pumping would occur in all parts of the membrane over a series of cycles.

The movement of S/cycle will decrease from a maximum value at the membrane surface to a minimum value at the center, so that there will be an average distance covered by S/pumping cycle. If we let the ratio of this distance to the thickness of the pumping zones be P_s, and the concentration of S be $[S_i]$ in the cytoplasm and $[S_c]$ at the center of the membrane, then the fluxes in the outer and inner zones will be respectively

$$F_0 = \left(\frac{[S_0]}{K + [S_0]} - \frac{[S_c]}{K + [S_c]}\right) N_p P_s W$$

$$F_i = \left(\frac{[S_c]}{K + [S_c]} - \frac{[S_i]}{K + [S_i]}\right) N_p P_s W$$

The total flux will be the sum of these or

$$F = \left(\frac{[S_0]}{K + [S_0]} - \frac{[S_i]}{K + [S_i]}\right) N_p P_s W \tag{6}$$

movement into the cell being positive.

Equations similar to (6) have been derived by a number of workers on a variety of premises[3] and shown to provide adequate quantitative descriptions for a number of examples of facilitated transport. The qualitative aspects of transport can be derived from eqn. (6), however, in the following ways: (a) With $[S_i] = 0$, increasing $[S_0]$ results in an increase in F but only to a maximum value which is defined by (6) as F max. $= N_p P_s W$. (b) F becomes zero only when $[S_0] = [S_i]$. (c) For a system in which $[S_0]$ is very large and $[S_i]$ initially zero but increasing, 2 cases may be distinguished. (1) When K is small, F will be large initially but drop off rapidly as $[S_i]$ exceeds K finally approaching zero while a considerable difference still exists between $[S_i]$ and $[S_0]$. (2) When K is relatively large, (*i.e.* the tendency to form complexes is small) the initial flux is smaller, but the system does not become saturated as readily and $[S_1]$ will approach a larger value here than in case (1) before the flux appears to be zero.

Both examples have been demonstrated in the case of red blood cell transport of sugars, glucose being an example of case (1) and sorbose of case (2)[4,5,24]. Table I shows, by the introduction of measured values of K into eqn. (6), the greater initial flux of dextrose as well as its more rapid decline with increasing $[S_1]$.

TABLE I

THE EFFECT OF $[S_1]$ ON THE FLUX OF SUGARS INTO HUMAN ERYTHROCYTES

Values calculated from eqn. (6) using LEFEVRES[4] values for K of approximately 0.01 M for dextrose and 1 M for sorbose. $[S_0] = 0.3\ M$.

$]S_1]$	F for dextrose	F for sorbose
0.00 M	0.97 N_pP_sW	0.23 N_pP_sW
0.01 M	0.47 N_pP_sW	0.22 N_pP_sW
0.03 M	0.22 N_pP_sW	0.20 N_pP_sW
0.10 M	0.06 N_pP_sW	0.14 N_pP_sW
0.15 M	0.03 N_pP_sW	0.10 N_pP_sW
0.20 M	0.02 N_pP_sW	0.06 N_pP_sW

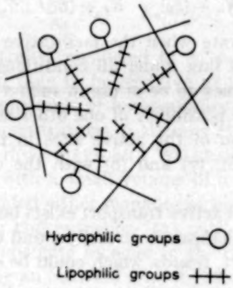

Fig. 2.

Passive transport: The movement of lipophilic compounds through the cell membrane is generally considered to be "passive" since their rate of transport is not dependent on structure directly but appears to be related to their oil–water partition coefficient, and their molecular size[25]. According to the present theory such compounds could only be transported if sites were present with which they could combine. Such sites probably are present in the form of micelles of lipophilic groups which could be formed in the contracting matrix by the attraction of a number of lipophilic side chains for each other. (Fig. 2). Lipophilic compounds could then partition between the aqueous phase and these micelles, and an expression for the flux could be obtained from eqn. (6) by letting K be the partition coefficient of the compound between water and the micelles.

Active transport: The treatment of active transport is essentially the same as for facilitated transport, the one difference being that during expansion the equilibrium constant K_e will have a value different to that during contraction K_c. Such sites must be present in one zone only, sites in the other zone being facilitative. When differential sites are present in the outer zone at a concentration N_a, the flux of material toward the center will be

$$F_0 = \left(\frac{[S_0]}{K_e + [S_0]} - \frac{[S_c]}{K_c + [S_c]}\right) N_a P_a W$$

giving for equilibrium.

$$[S_c] = \frac{K_c}{K_e} [S_0]$$

Since facilitative sites are present in the inner pumping zone, then at equilibrium, $[S_i]$ will be equal to $[S_c]$ and

$$[S_i] = \frac{K_c}{K_e} [S_0] \tag{7}$$

The direction in which transport is active will depend on the ratio of the constants, *i.e.* when $K_e > K_c$ net movement is outward while when $K_c > K_e$ the inside concentration exceeds the outer.

When the facilitative pumping mechanism is more rapid than the active, $[S_i] = [S_c]$ at all times and

$$F = \left(\frac{[S_0]}{K_c + [S_0]} - \frac{[S_i]}{K_e + [S_i]}\right) N_a P_a W \tag{8}$$

Eqn. (6) describes the flow rate when the facilitative transport is rate controlling while a general expression of flux under all conditions results from a combination of (6) and (8) but is too complex to be of use in such a qualitative discussion as this and is not included here. The possibility of one other arrangement of sites should be noted, this being the location of the active sites in the *inner* zone. The resulting expressions are similar to (6), (7) and (8) with the direction of active transport reversed.

Very little kinetic data on active transport exists but such results as are available indicate[6] that the active mechanism is reversible, and can be saturated in the same way as is facilitated transport, results which could be expressed by (8) as they were by (6).

Effect of inhibitors: Inhibitors might be expected to attack the transport mechanism at a number of levels. General enzyme poisons for example might destroy the mechanism directly by attacking enzymes in the membrane responsible for pumping, or they may have no effect on the pumping mechanism itself but penetrate into the cell destroying its internal metabolic processes and thus effecting the pumping mechanism indirectly. Membrane poisons could also exist which have a more specific affinity for the actual pumping processes while having no direct effect on the cell metabolism. Such inhibitors could conceivably inhibit only the contraction phase, in which case the matrix would remain in an expanded and highly permeable condition, (chemical lysis) or they might attack the expansion phase reducing overall penetrability of the cell. An example of this type of poison is the narcotic or physical poison which is considered to act by concentrating in the lipid layer by virtue of its high lipid solubility. Such compounds might also concentrate in the membrane micelles mentioned above, stabilizing and expanding them and thus increasing the lipophilic nature of the gel to the point where water entry is reduced and the swelling of the matrix no longer occurs. Other compounds might chemically attack certain points of attachment in the gel bringing about a state of lysis (*i.e.* by destruction of S–S-linkages). Finally there are specific poisons which combine either reversibly or irreversibly with certain of the transport sites. These have no effect on the overall membrane permeability but only inhibit the transport of a narrow range of compounds, *i.e.* those compounds having an affinity for the inhibited sites.

The case of competitive inhibition of facilitative sites may be treated quantitatively in the following way. If I is the inhibitor having a concentration $[I_0]$ outside the cell and $[I_1]$ inside the cell, and forming complex X_I with the sites, then we may write for the equilibrium conditions in the outer pumping layer

$$S + C_s \rightleftharpoons X_s, \qquad K_s = \frac{[S_0]\ [C_0]}{[X_s]_0},$$

$$I + C \rightleftharpoons X_I, \qquad K_I = \frac{[I_0]\ [C_0]}{[X_I]_0},$$

$$\text{and} \qquad n_p = [C_0] + [X_s]_0 + [X_I]_0$$

Similar expressions may be obtained for the inner pumping zone, and by solving for $[X_s]_0$ and $[X_s]_1$ we arrive as before, at an equation for the inward flux of substrate:

$$F_s = \left(\frac{[S_0]}{K_s + [S_0] + \frac{K_s}{K_I}[I_0]} - \frac{[S_1]}{K_s + [S_1] + \frac{K_s}{K_I}[I_1]} \right) n_p d_s W \qquad (10)$$

Competive inhibitors are generally considered to have a high affinity for the sites with which they combine and thus to be effective at relatively low concentration. Inhibition of transport by compounds of lower affinity can occur, however, if they are present in large enough concentrations. A striking example of this is provided by some recent investigations of ROSENBERG AND WILBRANDT[26]. These workers allowed red blood cells to equilibrate with a small volume of a low concentration (5.6 m*M*) of radioactive glucose, then added either mannose or non-radioactive glucose to this solution giving a much higher concentration (115 m*M*) of the nonradioactive compound. This resulted in an immediate increase in the radio-activity of the solution external to the cells (indicating an outward flux of radioactive sugar), followed by a decrease to the original value again as the inward flux of non-active material subsided. Thus, while a large concentration difference of the non-active species existed, there was uphill transport of the active.

Such a phenomenon would appear to be inexplicable by any stationary site theory but is, as ROSENBERG AND WILBRANDT have shown, a natural outcome of the operation of mobile sites. In terms of the present theory, which is basically a mobile site system, the outward flux of radio-active glucose into a solution having a large concentration of non-radioactive glucose will be given by eqn. (10) in which $K_s = K_I$, S refers to the radioactive glucose and I the non-active glucose, thus

$$F_s = \left(\frac{[S_0]}{K_s + [S_0] + [I_0]} - \frac{[S_1]}{K_s + [S_1] + [I_1]} \right) n_p d_s W$$

From this expression it can be seen that when $[I_0]$ is large and $[I_1]$ small or zero there is a large negative flux of S, whereas when $[I_0] = [I_1]$ the flux is normal (*i.e.* toward the region of lower $[S]$).

Examples of operation of the pump

Ionic distribution in cells: Despite the fact that the membrane of all cells is permeable to Na^+ and K^+, not all cells contain these ions to the same concentration as their environment. Thus some cells are able to concentrate K^+ while excreting Na^+ resulting in a high internal ratio of $[K^+]$ to $[Na^+]$, despite a low external ratio. This indicates the presence in the membrane of sites engaged in ionic transport. Such sites probably consist of a small concentration of permanently ionized groups which are attached to the matrix, and which are differential in nature, tending to complex sodium ions and release potassium during expansion, and reversing this preference during contraction. The membrane matrix thus acts as an ion exchange resin which alters its characteristic with each change in the direction of the solution flow. Due to the reciprocating nature of the pumping mechanism the sites need only show a slight preference for one ion over another during any given cycle. This small difference is then multiplied by succeeding cycles to give the required large differential.

Exchange diffusion: It was first shown by USSING[27] through the use of radioactive sodium that there is a rapid exchange between ions internal and external to the cell, even with no net ionic flux. This has been explained on the basis of a mobile carrier mechanism. According to the present theory, the normal pumping action results in molecules and ions present in the external solution and the cytoplasm constantly being brought into contact with molecules and ions attached to sites in the membrane, thus providing ample opportunity for exchange. Such exchange will occur not only through a facilitative mechanism but through an active transport mechanism as well.

Hydrogen ion excretion: The excretion of hydrogen ions produced during the metabolic cycle is probably one of the most important examples of active transport. The ability to perform this excretion is possessed to a very high degree by the oxyntic cells of the gastric mucosa[28] and by fermenting yeast cells[29], the latter even excreting ions into a medium having a pH of 1.5 while maintaining an internal pH of 6.5—a concentration ratio of 10^5. Such high concentration gradients may be sustained only in the presence of external potassium ions with which the hydrogen ions are exchanged.

Hydrogen ion pumping could be explained by the present theory by proposing the existence of a number of facilitative and differential sites in the membrane. The facilitative sites are probably nothing more than a low concentration of permanently ionized groups present in the *outer* pumping zone. The differential sites on the other hand might be acidic groups, attached to the protein matrix in the *inner* pumping zone, which have a lower dissociation constant during contraction (K_{ac}) than during expansion (K_{ae}). This change would result from alterations in molecular structure of that part of the matrix to which the acid group is attached. During expansion then, hydrogen ions from the cell interior would be swept into the membrane where the differential sites are present as potassium salts. As the matrix changes its nature and starts to contract, however, these sites would become weak acids taking up the hydrogen ions and releasing potassium. The hydrogens, now in combined form, would be carried toward the center by the contracting matrix and released there during the following expanding phase, while the potassium ions would be swept into the protoplasm. The facilitative sites in the outer pumping zone would allow exchange of hydrogen ions for potassium ions and result in an outward flow of hydrogen ions and an inward flow of potassium ions. As an alternative to the above mechanism, the active sites might be located in the outer pumping zone. However, this would require that they be in the ionized or polar form during the contraction or lipophilic phase, making this arrangement of sites less likely.

The wave-like nature of the pumping mechanism: To be consistent, any theory of cytoplasmic membrane function should explain the operation of *all* such membranes, even those of specialized cells such as excitable tissue. In compliance with this principle an attempt will be made below to show that the mechanism of propagation in nerve and muscle fibers is similar to and in fact may have been evolved from the pumping mechanism outlined above. If we agree to this similarity in advance, then an answer to the next question, that is how the pumping cycle is initiated, is suggested, since if the nerve impulse is propagated as a wave of activity passing over the nerve membrane, the same might be true of the pumping mechanism. More specifically then, we might consider the transport mechanism as resulting from the action of a series of self-propagated waves of expansion and contraction passing through the membrane gel.

As suggested above such waves are probably biochemical in nature resulting from an increase in osmotically active species within the gel matrix. Such species probably are produced and subsequently destroyed by enzyme action. The steps involved in the propagation then would be as follows: the contracted gel in its excitable condition would contain pockets of substrate separated from enzyme sites by channels too small for the substrate molecules to traverse. However, the distance separating enzyme and substrate would be very small and the probability is that sooner or later one of the channels would be widened as a result of Brownian movement of the protein chains, thus letting the substrate through to the enzyme site in a very localized region of the gel. The splitting of this substrate by the enzyme would bring about an increase in osmotic pressure with a consequent influx of water and expansion of the gel. This disturbance would be highly localized but would cause a distortion and movement of water in the surrounding matrix, so that pores in this region would be widened allowing further substrate to reach enzyme sites and causing the expansion wave to spread until it reaches a region in which a refractory (non active) condition exists. With the appearance of the osmotically active species, secondary processes would be initiated which would decrease the number of osmotically active species and bring about contraction of the gel. Recovery processes would then build up the concentration of the initial substrate, returning the gel to its excitable condition. The resting gel then is in a metastable state since any disturbance of the gel structure could act as a point of origin from which a wave of action could spread throughout the membrane. A number of such points would arise spontaneously in various parts of the membrane and cause a number of waves to pass over the surface of the cell in different directions.

This might well be the explanation of the optical phenomenon known as "flickering" in the red blood cells, a phenomenon observed by a number of workers who have described it as a scintillating movement passing across erythrocyte cells in a fashion somewhat reminiscent of the movements produced by the wind blowing over a field of wheat. TOMPKINS[30] has reported that this phenomenon appears most clearly near the center of the concave side of the cell, swirling either circumferentially or radially from the center. These fluctuations are different to Brownian movements in that they are more rapid, rhythmic and systematic. BLOWERS, CLARKSON AND MAIZELS[31] have demonstrated a number of similarities between the occurrence of flickering and of active cation transport. Both phenomena were found to be constrained by changes away from the normal cell shape and by agencies inhibiting glycolysis, but practically unaffected by respiratory inhibitors. Both operate over the same pH range and seem to be energized by the same sugars. In view of this apparent interrelationship these workers suggested the possibility that the flicker phenomenon is an expression of the activity of carrier molecules engaged in active transport. The present theory also suggest this relationship since changes in optical properties might be expected to accompany changes in hydration which sweep through the membrane gel in an oscilliatory fashion. Thus the flicker phenomenon may well be a visual manifestation of the osmotic pumping mechanism.

Transmission in excitable tissue

The wave of excitability passing through the membrane of a nerve axon results in 2 changes, one electrical and the other related to ionic fluxes[32–34]. As it passes a given point on the membrane, the potential across the membrane at this point, which is 10 to 100 mV negative on the inside in a resting nerve, Fig. 3(a), rises very rapidly to a peak called the "action potential" (b) then drops to a potential slightly below the resting potential (c) from where it slowly returns to its original value. The electrical change is accompanied by a movement of ions, Na^+ moving in to the cell and K^+ moving outward. This is the reverse of the normal movement occurring during quiescent periods which tends to concentrate K^+ on the inside at the expense of Na^+.

These observations are usually explained on the assumption that the membrane in its resting condition has a very high degree of permeability to K^+ but not Na^+, while during transmission the Na^+ permeability is increased considerably. Thus the system:

external solution | membrane | axoplasm

acts as a K^+ concentration cell at rest and a Na^+ concentration cell during periods of activity[35]. No explanation is offered by this theory, however, for the ability of the membrane to alter its selectivity so drastically in favor of Na^+ during excitation.

An alternate explanation arises from the present theory and will be presented briefly here. The wave of excitability is considered to be similar to, and probably derived from the normal pumping motion but independent of it, requiring a greater degree of stimulation and resulting in a more extensive and rapid expansion, brought about by a different enzyme-substrate system. Since the osmotically active species arises and disappears as a result of 2 consecutive reactions the concentration of this species, and hence the swelling of the gel will follow a time course similar to curve B Fig. 3. During the rising portion of this curve Na^+ from the outer solution will be swept into the gel to a much greater depth than during normal pumping and allowed to exchange with K^+ on sites well within the membrane. The K^+ thus displaced will be swept out as the gel shrinks, so that the net result is uptake of Na^+ and loss of K^+. At the same time, the more open structure of the membrane would explain the drop in its resistance observed during excitation.

The electric characteristics of the impulse could be explained on the assumption that a number of fixed anionic sites line the pores in the outer pumping zone or in the outer lipid layer. These could be for example, carboxyl groups of long chain fatty acids. The movement of solution through such pores would result in the production of a streaming potential which would make the inside of the cell more positive during uptake and negative during expulsion of the water. Since the streaming potential is proportional to the velocity of flow, a plot of the first derivative of curve B should give a curve describing the nature or the potential changes. Curve A represents such a plot, showing as we have already stated, the electrical characteristics of the nerve impulse.

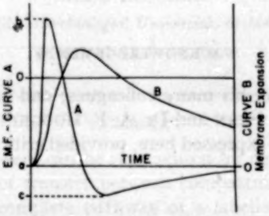

Fig. 3.

Water transport

Finally the wave nature of the pumping mechanism may be used to explain active water transport. Many cells have the ability to function normally despite a large osmotic pressure difference between their own cytoplasm and the solution in which they live. Other cells, such as those located in plant roots and animal kidney, are able to move water against a pressure gradient. In all such cells a mechanism must exist which is able, through the expenditure of metabolic energy, to produce and maintain an osmotic difference across the cytoplasmic membrane. The osmotic pump in its usual form is unable to accomplish mass transfer of water since the uptake and expulsion of water by the membrane is considered on the average, to occur simultaneously in both pumping zones. Suppose, however, that such symmetry does not exist and that, for example, expansion of the inner pumping zone does not commence until the expansion of the outer zone is complete, giving rise to pumping waves of the type shown diagramatically in Fig. 4. The uptake of water by the expanding outer zone will be almost entirely from the outside solution (as indicated by the arrow at A, Fig. 4),

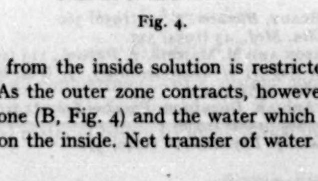

Fig. 4.

since the flow of water from the inside solution is restricted by the density of the contracted inner zone. As the outer zone contracts, however, some water will move from it into the inner zone (B, Fig. 4) and the water which was transferred between the 2 zones is retained on the inside. Net transfer of water in the opposite direction will occur when the expansion of the inner zone precedes that of the outer zone, so that we have a system capable of water movement in either direction. Some backward flow of water will occur of course, since even in its contracted state the membrane is not impervious to water, however, this may be countered by a continuous repetition of the pumping cycle.

ACKNOWLEDGEMENTS

The author wishes to thank his many colleagues; and in particular Professor J. F. Danielli, Professor W. O. Fenn and Dr. A. F. Hodgkin, who, while not necessarily agreeing with the concepts expressed here, provided criticisms helpful in the development of this work.

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BIOCHIMICA ET BIOPHYSICA ACTA

SYNTHESIS OF A MULTICOMPARTMENTED BIOLOGICAL MODEL

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(Received April 1st, 1959)

SUMMARY

A model of a biological system can be represented by defining the compartments of the system and the rates of transfer between compartments. The *flow diagram* is a net which represents the complete pathway of a labelled material flowing through the compartments. In this paper some rules are formulated to obtain, from the flow diagram, the *transfer function* of a system, *i.e.* a function which permits the fate of the labelled material in one compartment to be calculated either from the fate in another compartment or from the input of the labelled material in the system.

INTRODUCTION

A model of a biological system is constructed by defining the compartments and the rates of transfer between compartments[1]. A compartment is a component of the system characterized by the fact that on addition of the same component mixing is quicker than the transfer of the same component to the rest of the system. The rate of transfer from one compartment to another is that fraction of one compartment which enters another in unit time.

General equation of a system

Let k_{ab} be the rate of transfer from compartment a to compartment b and K_a the rate of transfer from compartment a to all other compartments: $K_a = \Sigma_i k_{ai}$, the sum being extended over all compartments connected with compartment a. In this paper only constant k's will be considered; this is the case when first order reactions occur in a compartment or when the compartment is in dynamic equilibrium[2].

If compartment i contains at time t an amount X_i of labelled material, then

$$\mathrm{d}X_i/\mathrm{d}t = \Sigma_j k_{ji}X_j - K_iX_i,$$

the sum being extended over all compartments flowing directly into compartment i.

Calling x_i the Laplace transform of X_i[3], then, for all compartments in which $X = 0$ for $t = 0$:

$$sx_i = \Sigma_j k_{ji}x_j - K_ix_i$$

or

$$x_i = \Sigma_j k_{ji}x_j/(s + K_i) \tag{1}$$

Eqn. (1) holds for all compartments of a system, including the compartment c into which the labelled material is first introduced, if a hypothetical compartment O, representing the labelled material before its introduction into the system, is included.

Flow diagram

A net can be constructed, composed of nodes, corresponding to the compartments of a system, connected by oriented arms; the generical node corresponding to compartment i has the value x_i, and the generical arm connecting node x_j to node x_i has the value $k_{ji}/(s + K_i)$. The compartment O is represented by node x_0, which is connected to node x_c by arm $1/(s + K_c)$. Such a net can be called a ***flow diagram***, as it will represent the complete pathway of the labelled material flowing through it[4]. From eqn. (1) the fundamental property of the flow diagram follows:

"Each node of the net is equal to the sum of the products of the nodes flowing into it and their respective arms."

If in a system no labelled material is fed back into any of the preceding compartments, or in the corresponding flow diagram no arms form a closed loop, the system or the diagram is then called of ***zero order***. If some feedback is present, ***essential nodes*** may be defined as those nodes that must be eliminated in order to interrupt all feedback loops: by eliminating a node it is meant that all arms leaving it must be disregarded. The order of a flow diagram is the minimum number of essential nodes.

Transfer function

The chief problem in the synthesis of a model is the determination of the fraction x_b/x_a, called the ***transfer function*** from compartment a to compartment b. To this purpose the flow diagram may be simplified by suppressing some nodes and altering the value of the arms in such a way that the value of the remaining nodes, calculated by means of the fundamental property, is not altered.

The flow diagram can be simplified by the following rules, which are a direct consequence of the fundamental property: (a) The nodes that receive no contribution from x_a, or give no contribution to x_b, and the arms diverging from these nodes can be eliminated; (b) Two tandem arms can be substituted by a single arm equal to the product of these arms, and the intermediate node suppressed; (c) Two parallel arms can be substituted by a single arm equal to the sum of these arms; (d) An arm entering a node can be substituted by arms entering all nodes following it, each new arm being equal to the product of the original arm and the arm connecting the previous end to the new one.

With these rules, all non-essential nodes different from x_a and x_b can be eliminated; in non-zero order diagrams, the rules are used until a feedback loop is reduced to a ***self-arm***, *i.e.* an arm starting and ending in the same node.

Essential diagram

A diagram reduced by these rules to a minimum number of nodes is called an ***essential diagram***, as it contains only the two extreme nodes and essential nodes, if any.

The order of the essential diagram is not greater than the order of the complete flow diagram.

The essential diagram of order p is composed, in general, of an arm connecting node x_a to node x_b directly, of p arms connecting the first node x_a to each of the essential nodes, of p arms connecting each of the essential nodes to the last node x_b, of $2\cdot\binom{p}{2}$ arms connecting the essential nodes with each other in all possible ways, and of p self-arms. Of course, some arms can be null, and either x_a or x_b, or both, can be essential nodes.

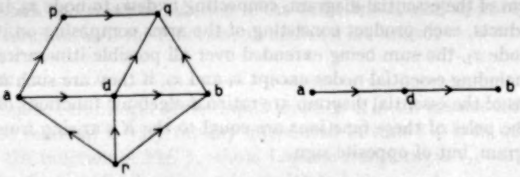

Fig. 1. Node r does not receive any contribution from a; nodes p and q do not give any contribution to b; they can be suppressed following rule (a).

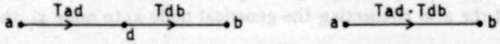
Tad
Tdb
a
d
b
Tad · Tdb
a
b

Fig. 2. The arms T_{ad} and T_{db} are substituted by arm $T_{ad} \cdot T_{db}$, and node d is suppressed, following rule (b).

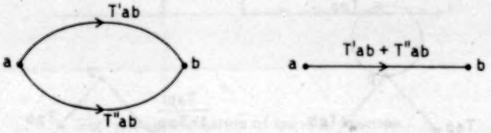
T'ab
a
b
T"ab
T'ab + T"ab
a
b

Fig. 3. The arms T'_{ab} and T''_{ab} are substituted by arm $T'_{ab} + T''_{ab}$, following rule (c).

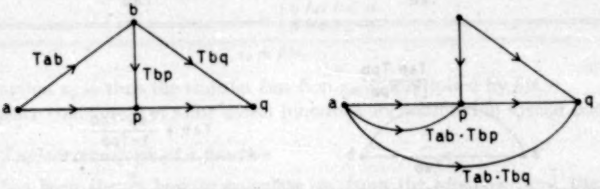
b
Tab
Tbp
Tbq
a
p
q
a
p
q
Tab · Tbp
Tab · Tbq

Fig. 4. Arm T_{ab}, connecting node a to node b, is substituted by arm $T_{ab} \cdot T_{bp}$, connecting a to p, and by arm $T_{ab} \cdot T_{bq}$, connecting a to q, following rule (d).

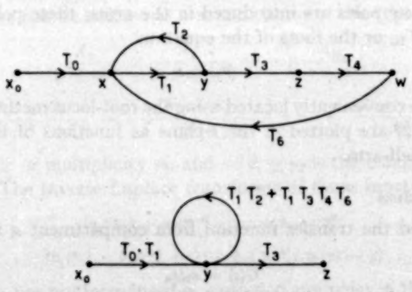
T2
T0
x0
x
T1
y
T3
z
T4
w
T6
T1 T2 + T1 T3 T4 T6
T0 · T1
T3
x0
y
z

Fig. 5. Complete flow diagram and essential diagram from node x_0 to node z of the thyroid model by ODDIE AND SCOTT[5].

Each arm of the essential diagram, connecting node x_i to node x_j, is the sum of a series of products, each product consisting of the arms composing an itinerary from node x_i to node x_j, the sum being extended over all possible itineraries between the nodes, not including essential nodes except x_i and x_j, if they are such nodes.

The arms of the essential diagram are rational algebraic functions of the complex variable s; the poles of these functions are equal to the K's arising from the arms of the flow diagram, but of opposite sign.

Reduction of the essential diagram

A self-arm can be eliminated from the essential diagram simply by multiplying all arms entering it by $1/(1-T_{ii})$, T_{ii} being the value of the self-arm. In effect if T_{ji} is the value of the arm connecting the generical node x_j to node x_i, then

$$x_i = \Sigma_j T_{ji} x_j + T_{ii} x_i$$

and hence

$$x_i = \Sigma_j T_{ji} x_j/(1 - T_{ii})$$

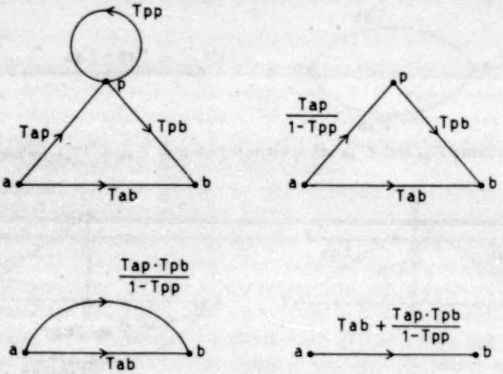

Fig. 6. Removal of a self-loop from an essential diagram of order one, and step by step reduction to a single arm equal to the transfer function.

In this way new poles are introduced in the arms; these poles are the zeros of the function $1 - T_{ii}$, or the roots of the equation

$$T_{ii} = 1 \tag{2}$$

These roots are conveniently located using the root-locus method[6]; by this method the roots of eqn. (2) are plotted in the s-plane as functions of the products of the k's present in the self-arm.

Use of transfer function

If $G(s)$ is called the transfer function from compartment a to compartment b, the equations

$$G(s) = x_b/x_a$$

and

$$x_b = x_a \cdot G(s)$$

serve to calculate the transfer function if the functions x_a and x_b are known, or to calculate the function x_b if the functions x_a and $G(s)$ are known.

Various inputs

x_o, the Laplace transform of the input function X_o, can assume different forms. If the labelled material is very rapidly introduced in time $t = 0$ in quantity a, X_o is defined as the function of Fig. 7, whose Laplace transform is $x_o = a$. Function x_a is thus simply the transfer function x_a/x_o multiplied by a.

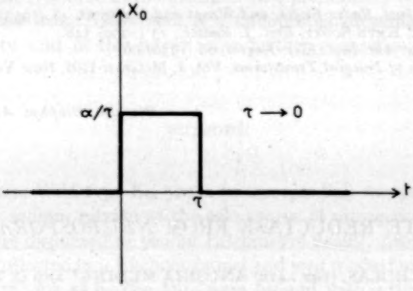

Fig. 7. Representation of impulse function.

If the labelled material is introduced at a constant rate β, then

$$X_o = \begin{cases} 0 \text{ for } t < 0 \\ \beta \text{ for } t > 0 \end{cases}$$

$$x_o = \beta/s$$

Function x_a is thus the transfer function x_a/x_o multiplied by β/s.
Laplace transforms of some useful functions are collected in special books[7].

Inverse Laplace transform of a function

It has been shown how to calculate x_a from the input x_o and the transfer function x_a/x_o. The final problem is to find the inverse Laplace transform of the function x_a.

The function x_a can be written as a sum of terms of the type

$$\frac{A_1}{(s+a)^m} + \frac{A_2}{(s+a)^{m-1}} + \cdots + \frac{A_m}{s+a};$$

$$\frac{B_1 s + C_1}{[(s+\beta)^2+\gamma^2]^n} + \frac{B_2 s + C_2}{[(s+\beta)^2+\gamma^2]^{n-1}} + \cdots + \frac{B_n s + C_n}{(s+\beta)^2+\gamma^2};$$

$-a$ is the real pole of multiplicity m, and $-\beta \pm \gamma j$ is the complex conjugated pole of multiplicity n. The inverse Laplace transforms of these functions are of the type

$$(a_1 t^{m-1} + a_2 t^{m-2} + \cdots + a_m)\, e^{-at};$$

$$(b_1 t^{n-1} + b_2 t^{n-2} + \cdots + b_n)\, e^{-\beta t} \cos(\gamma t - \varphi)$$

If the input x_o has not introduced or cancelled any poles in the transfer function, the exponentials of the function X_a are the real parts of all poles of the transfer function. These are the poles of the arms of the essential diagram, *plus* the roots of the eqns. (2) of the self-arms, *minus* the poles of the self-arms, which may be in common with other arms.

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A NITRITE REDUCTASE FROM *NEUROSPORA CRASSA*

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(Received April 23rd, 1959)

SUMMARY

Nitrite reductase has been purified, over 50-fold from ordinary felts of *Neurospora crassa*, macroconidial wild type Em 5297a. The enzyme is a DPNH-dependent flavoprotein containing FAD, Fe and Cu and –SH groups. Copper may act by coupling the flavin component of the enzyme to nitrite since an external supply of Cu^{1+} reduced NO_2 non-enzymically; this reduction increased in the presence of the enzyme. The role of iron in the enzyme is not known since neither Fe^{2+} nor reduced cytochrome *c* will reduce nitrite. The enzyme is also dependent on Mg and pyridoxine for maximal activity. Although the addition of pyridoxine or pyridoxal or pyridoxal phosphate reactivated the enzyme in crude extracts of the pyridoxine-requiring mutant deficient in the vitamin, there was no similar effect with the purified enzyme. A possible explanation for this phenomenon is presented. The reduction product inhibited the enzyme and it is shown that the addition of hyponitrite but not of hydroxylamine depressed nitrite reductase activity.

INTRODUCTION

Nitrite reductase was first determined in cell-free extracts of *Bacillus pyocyaneus* by Yamagata[1]. Subsequently Taniguchi *et al.*[2] found the enzyme in *Bacillus pumilis*

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using reduced methylene blue as the electron donor. NASON, ABRAHAM AND AVERBACH[3] showed that nitrite is enzymically reduced to ammonia in extracts of *Neurospora crassa* and in those of soya-bean leaves. The production of ammonia from nitrite in the leaf extracts was stimulated by adding manganese but this effect could have occurred at any of the enzymic steps between nitrite reduction and ammonia production. SILVER AND McELROY[4] have also determined nitrite reductase activity in biochemical mutants of *Neurospora* and more recently SPENCER, TAKAHASHI AND NASON[5] have characterized a nitrite reductase from *Azotobacter agile* grown with or without nitrate as the N source in the medium. In a preliminary report[6] it was shown that nitrite reductase from *Neurospora* is a flavoprotein dependent on Cu and Fe for maximal activity and in this paper some further properties of the enzyme are described.

METHODS

Enzyme source

Neurospora crassa Wild type E_m 5297a (macroconidial) was grown in a modified Fries medium with sodium nitrate as the sole source of nitrogen; 100-ml lots of the culture solution were dispensed in 500-ml Erlenmeyer flasks[7]. After 4 days growth in the dark, the felts collected in a Büchner funnel and well washed with deionised water were stored at — 17° for 12 h. The felts were ground with 4 times their weight of 0.1 *M* phosphate buffer (pH 7.5) and then in a Ten Broeck glass macerater at 0°. The homogenate was centrifuged at 3,000 × *g* for 15 min at 4° and the supernatant solution was used as the source of the crude enzyme. This resulted in approximately 80 % extraction of the enzyme.

The enzyme was also prepared in a similar way from a pyridoxine-requiring mutant of *Neurospora crassa* (kindly supplied by Dr. M. MITCHELL, California Institute of Technology, U.S.A.) grown on the Fries media supplemented with 0.1 mg pyridoxine/l.

Assay of nitrite reductase

The enzyme assay included: 15 µmoles phosphate (pH 7.5), 0.02 µmole $NaNO_2$, 0.1 ml boiled pig heart, 0.8 µmole DPNH, 0.10 ml enzyme (14 mg protein/ml) in a final volume of 1 ml. Control assays were always included in which DPNH was omitted and others in which the enzyme was boiled. After incubating for 10 min at 25°, the following additions were made: 0.1 ml 1 % v/v acetaldehyde and crystalline alcohol dehydrogenase (prepared from yeast) and after standing for a further 2 min 0.1 ml *M* zinc acetate and 3 ml cold 90 % v/v ethanol. After each addition the solutions were well agitated with glass rods. After centrifuging at 3,000 × *g* at 4°, nitrite was determined in 0.5 ml of the supernatant solution by adding 0.5 ml 1 % w/v sulphanilamide in *N* HCl and 1 ml 0.01 % w/v N-(1-naphthyl) ethylenediamine dihydrochloride.

Determination of flavin constituents

The purified enzymes were boiled for 10 min and then centrifuged at 3,000 × *g*. The flavin constituents in the supernatant solution were separated by means of paper chromatography[8] and by paper electrophoresis[9].

Iron and copper determinations

The two metals were determined in various protein fractions after digesting them in nitric and perchloric acids[10]. Diphenyldithiocarbazone was the reagent used for copper[11] and the α,α-dipyridyl method for iron[12].

The iron contents of various fractions were also determined independently by the radioassay method. For this purpose, iron was removed from the basal culture solution by the 8-hydroxyquinoline method[13]. [^{59}Fe]ferric chloride (200 μg Fe/1.25 μC ^{59}Fe/l was then returned to the culture solution which was dispensed as described above. Tracer iron was determined with a scintillating counter[14].

Cofactors and other reagents

DPN 98% pure (Sigma Chemical Company, St. Louis, U.S.A.) was enzymically reduced by the alcohol dehydrogenase method[15]. Boiled pig heart was prepared by centrifuging a water extract of acetone powder of pig heart at 15,000 × *g*, boiling the supernatant solution for 15 min and centrifuging again at 6,000 × *g* for 10 min. Riboflavin 5-phosphate (FMN) and flavin-adenine dinucleotide (FAD) each 98% pure were obtained from Sigma Chemical Company, U.S.A. A sample of 2 heptyl-4 hydroxyquinoline-N-oxide was generously provided by Dr. J. W. CORNFORTH, National Institute for Medical Research, Mill Hill, London. Haemin, containing 8.46% Fe, was obtained from the British Drug Houses Ltd., Poole, England. Freeze-dried preparations of *Streptococcus faecalis* R, deficient in pyridoxine, for the assay of pyridoxal phosphate and pyridoxal by the tyrosine decarboxylase method[16] were kindly supplied by Professor D. D. WOODS, F.R.S., Department of Microbiology, Oxford University.

Micronutrient deficiencies: Individual deficiencies of Fe, Cu, Mn, Mo and Zn were produced in the fungus by methods described previously[13].

Protein was determined by Folin's method as modified by LOWRY *et al.*[17]. Enzyme fractions containing ammonium sulphate were dialysed against running water to remove ammonium ions before determining protein.

RESULTS

Fractionation of enzyme: The fractionation of the enzyme shown in Table I resulted in a 50-fold purification. The purified enzyme is not stable since its activity was halved overnight when kept at — 17° and is deactivated after dialysis against water or a variety of buffers.

Substrate saturation: The dissociation constant (K_m) of the enzyme-nitrite complex was calculated from the saturation curve to be $1 \cdot 10^{-4}$ moles/l. DPNH is a more effective electron donor than TPNH at equivalent amounts. The maximal activity for DPNH or TPNH was $1.2 \cdot 10^{-3}$ moles/l.

Flavin requirements: The inhibition of the enzyme by atabrine (mepacrine) was reversed by FAD as shown in Table II. The addition of boiled pig heart, FMN or FAD to the purified enzyme increased its activity. FMN was as effective as FAD in stimulating nitrite reduction. The K_m for both flavins was $5 \cdot 10^{-5}$ moles/l. The native flavin in the purified preparations was identified as FAD both by paper chromatography and by paper electrophoresis.

pH optimum: Maximal activity was reached at about pH 7.6 in phosphate buffer

TABLE I

PURIFICATION OF NITRITE REDUCTASE FROM *N. crassa*

Mycelia weighing 60 g (fresh wt.) from about 20 flasks were used for purification of the enzyme. The mycelium was homogenised in 0.01 *M* phosphate (pH) and the supernatant solution left after centrifuging the homogenate at 4000 × *g* was fractionated as described in the text. The activityof the enzyme was measured in the usual reaction mixture.

	Total protein (mg)	*Total units*	*Protein (mg/ml)*	*Specific activity mμmoles NO_2 reduced/mg protein/10 min*	*Percentage recovery*
1. Supernatant solution	1050	10,800	10.5	10.3	100
2. Calcium phosphate gel added to (1) (25 mg/100 mg protein). Stand for 5 min at 0°. Centrifuge at 4000 × *g*. Gel washed twice with 0.01 *M* phosphate (pH 7.4) and eluted with 0.05 *M* phosphate (pH 7.4). Activity of supernatant solution.	104	9,000	1.3	86.3	83
3. Protamine sulphate added to (2) (15 mg/100 mg protein). Stand for 15 min. Centrifuge at 4,000 × *g*. Activity of supernatant solution.	56.5	9,000	0.70	159	83
4. $(NH_4)_2SO_4$ precipitate of (3), between 40 and 60% saturation, dissolved in 0.01 *M* phosphate (pH 7.4).	43.5	8,100	0.60	186	75
5. Calcium phosphate gel added to (4) (25 mg/100 mg protein). Stand 15 min, centrifuged at 4,000 × *g*. Gel washed twice with 0.01 *M* phosphate (pH 7.4) and eluted with 0.05 *M* phosphate (pH 7.4). Activity of eluate.	14.4	3,200	0.24	220	30
6. $(NH_4)_2SO_4$ precipitate of (5), between 50 and 60% saturation, dissolved in 0.1 *M* phosphate (pH 7.4).	4.4	2,300	0.11	523	21

TABLE II

INHIBITION OF NITRITE REDUCTASE BY ATABRINE AND ITS REVERSAL BY FAD

The activity of the enzyme was measured in the usual reaction mixture (see METHODS) to which 2 μmoles of atabrine (mepacrine) were added with, and without, 0.01 and 0.02 μmoles FAD. The enzyme was incubated for 10 min at 25° with the atabrine, with or without FAD, prior to enzyme assay.

Compounds added to 0.05 ml enzyme containing 14 mg protein/ml	*% Inhibition*	*% Reversal of inhibition*
2 μmoles atabrine or (mepacrine)	61	0
2 μmoles atabrine + 0.01 μmoles FAD	28	33
2 μmoles atabrine + 0.02 μmoles FAD	8	53

with 40% activity in sodium acetate–acetic acid buffer (pH 4.0) and 70% activity at pH 11.0 in glycyl–glycine buffer.

Enzyme concentration: An increase in the amount of enzyme added produced a proportional increase in nitrite reduction.

Time factor: Incubation of the assay mixture for various time intervals resulted in a falling off of activity after 15 min, which indicates that the product of the reaction inhibited the enzyme. When 0.5 μmoles of sodium hyponitrite or 8 μM hydroxylamine was added to the enzyme the former inhibited the enzyme, whereas the hydroxylamine was without effect even at a concentration twenty times greater than that of hyponitrite.

Effect of inhibitors: The general metal inhibitors KCN, 8-hydroxyquinoline, Na-EDTA, depressed enzyme activity, as did sodium diethyldithiocarbamate, salicylic acid and thiourea, diquinolyl, α, α-dipyridyl and *o*-phenanthroline, which are more specific inhibitors of Fe and Cu. Hydrazine sulphate inhibited the enzyme completely. The haem-inhibitors, 2-heptyl-4-hydroxyquinoline-N-oxide, naphthaquinone and urethane markedly reduced the activity of nitrite reductase.

The inhibition by *p*-chloromercuribenzoate which forms stable mercaptides with one available protein thiol group, was reversed completely by glutathione but that of iodosobenzoate, which requires 2 thiol groupings for its more rapid and complete action, could not be reversed.

Effects of metal deficiencies: The enzyme activity of extracts of felts deficient in Fe, Cu, Mn, Mo and Zn respectively are given in Table III. A deficiency of either Mg or Fe or Cu or Zn markedly depressed its activity. Experiments in which extracts of

TABLE III

EFFECTS OF METAL DEFICIENCIES

Specific activities of nitrite reductase and growth data as % of the values for normal felts. Enzyme assay as in METHODS.

Treatments		*Omit Mg*	*Omit Fe*	*Omit Cu*	*Omit Mo*	*Omit Zn*	*Omit Mn*
Enzyme	Expt. 1	34	9	52	100	60	102
	Expt. 2	30	18	34	104	64	100
Weight of felts	Expt. 1	40	10	57	42	60	41
	Expt. 2	40	33	46	51	40	40

normal felts were mixed with equal volumes of those from felts deficient in Fe or Cu or Mg showed that there was neither an inhibitor in the deficient ones nor an activator in the normal felts. In Zn-deficient felts however an inhibitor was detected[6]. A deficiency of Mo or Mn was without effect on the enzyme.

Effects of graded amounts of iron and copper in the medium: The results of enzyme assays in felts given increasing amounts of Fe and Cu respectively in the medium, from deficiency to sufficiency levels, showed that enzyme activity was proportional to the Fe supplied over the range 0–230 μg/l and for Cu, 0–20 μg/l. Similar results were obtained with Mg.

The results of adding iron compounds to extracts of normal and Fe-deficient felts and copper salts to those from Cu-deficient felts are shown in Table IV. The addition of Fe^{3+} as ferric chloride markedly stimulated enzyme activity but haemin resulted in only a small activation and mammalian cytochrome *c* was without effect. The enzyme in extracts from control felts was not significantly affected by the iron compounds. The addition of Cu at 1 μg/mg protein resulted in maximum stimulation of enzyme activity in extracts from Cu-deficient felts. In extracts from control felts the amounts of Cu above 0.15 μg/mg protein depressed the enzyme. The addition of Mg, however, did not reactivate the enzyme in extracts of Mg-deficient felts.

TABLE IV

EFFECTS OF ADDING Fe COMPOUNDS OR $CuCl_2$ *in vitro* ON THE ACTIVITY OF NITRITE REDUCTASE

Enzyme activity was measured after incubating the above compounds for 10 min at 25° in the usual reaction mixture (see METHODS) prior to assay.

Compound added/mg protein in crude extract	*% Activation (A) or % inhibition (I) of enzyme from control felts*	*% Activation of enzyme Iron compounds added to extracts from Fe-deficient felts and $CuCl_2$ to those from Cu-deficient felts*
0.2 μg Fe^{3+} ($FeCl_3$)	0	100
0.4 μg Fe^{3+} ($FeCl_3$)	30 (A)	110
0.8 μg Fe^{3+} ($FeCl_3$)	20 (A)	110
0.2 μg haemin-Fe	20 (A)	60
0.4 μg haemin-Fe	20 (A)	60
0.004 μmoles cytochrome *c*	0	0
0.08 μmoles cytochrome *c*	0	0
0.05 μg Cu^{2+} ($CuCl_2$)	0	28
0.15 μg Cu^{2+} ($CuCl_2$)	45 (I)	30
0.20 μg Cu^{2+} ($CuCl_2$)	60 (I)	30
0.50 μg Cu^{2+} ($CuCl_2$)	65 (I)	70
1.10 μg Cu^{2+} ($CuCl_2$)	80 (I)	100
1.60 μg Cu^{2+} ($CuCl_2$)	80 (I)	70

Metal constituents of the enzyme: The Cu and Fe contents of various enzyme fractions were related to their activities. The results for Cu are illustrated in Fig. 1. It is clear that Cu concentrates in the most active fractions of the enzyme. A similar result was obtained for iron using the chemical and radioassay method; fraction (6) Table I, contained 0.03 μg Fe/mg protein. Although the enzyme formation is dependent on Mg this metal did not accumulate in the purified enzyme.

Role of metals in the enzyme: Experiments were made to determine whether or not an exogenous supply of Cu^{1+} or Fe^{2+} would act as electron donors for the enzymic or non-enzymic reduction of nitrite. The results given in Table V show that when a small amount of Cu^{1+}, comparable to that in the enzyme, was added to the purified enzyme and DPNH, nitrite reduction increased over the amount resulting from the chemical reduction of NO_2^- by Cu^{1+}.

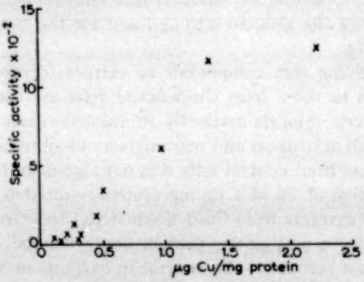

Fig. 1. Relation between Cu content of protein fractions and their enzyme activity. Specific activity: mµmoles NO_2 reduced/10 min/mg protein.

TABLE V

Cu^{1+} AS AN ELECTRON DONOR FOR THE ENZYMIC AND NON-ENZYMIC REDUCTION OF NITRITE

Enzyme assay for (1) as in METHODS. For (2 to 4), Cu_2Cl_2 (0.01 µg Cu^{1+}/0.2 ml) was also used as an electron donor in the assay. Cu_2Cl_2 was prepared by boiling $CuCl_2$ with copper turnings in an excess of 10 *N* HCl and pouring the solution into a tenfold excess of distilled water. The precipitate was well washed by decanting it in turn with water, acetone and ether in an atmosphere of CO_2 and dried in a stream of CO_2 at 50–60°. The pure white product which was preserved in CO_2 in a sealed tube, was dispensed in CO_2 free *N* HCl (1 mg Cu^{1+}/ml). When required for assay this solution was diluted with boiled glass distilled water (0.01 µg Cu^{1+}/0.1 ml), kept under CO_2, and was used immediately in the assay in the well of the Thunberg tubes. The other reagents were put in the sidearm and after evacuation, the contents were mixed and incubated for 10 min before testing for nitrite reduction.

	mµmoles NO_2 reduced/ml purified enzyme (fraction 6, Table I)			
	(1) *DPNH* *FAD* *NO_2* *Enzyme*	*(2)* *Cu^{1+}* *FAD* *NO_2* *Enzyme*	*(3)* *DPNH* *Cu^{1+}* *FAD* *NO_2* *Enzyme*	*(4)* *Cu^{1+}* *FAD* *NO_2* *Boiled enzyme*
Expt. 1	92	41	180	15
Expt. 2	96	25	160	15

Similar experiments with Fe^{2+} and reduced cytochrome *c* showed that neither reduced nitrite either in the presence or absence of the enzyme.

Pyridoxine requirement: An increase in pyridoxine in the medium from 0–50 µg was paralleled by an increase in nitrite reductase activity in the felts of a pyridoxine-requiring mutant of *N. crassa*. The addition of pyridoxine *in vivo* to pyridoxine-deficient felts stimulated the formation of the enzyme within a few minutes of adding it. This result is different to the slow induction of the enzyme in felts deficient in Cu or Fe or Mg when the appropriate metal was returned *in vivo*.

The enzyme was activated *in vitro* in crude extracts of pyridoxine-deficient felts by returning either pyridoxine, pyridoxal or pyridoxal phosphate. The purified enzyme, however, was not stimulated by these factors. Neither pyridoxal nor pyridoxal phosphate was detected in purified fractions of nitrite reductase by the tyrosine decarboxylase method[16].

Nitrite accumulated in the media of the pyridoxine requiring mutant of *Neurospora* deficient in pyridoxine and this was utilized when pyridoxine was returned to the culture solution.

Contrary to the results of SILVER AND MCELROY[4], who found that their *Neurospora* mutant required pyridoxine only when grown on nitrite or nitrate, but not when ammonium was the only nitrogen source, the mutant which we have used has a similar pyridoxine-requirement for maximal growth, whether it is grown on an equivalent amount of nitrate or ammonium ions.

DISCUSSION

That the reduction of nitrite in *Neurospora crassa* is dependent on flavin, Fe, Cu and DPNH for maximal activity[6] is here confirmed by using another assay in which nitrite reduction was determined instead of ammonia production. The same metal constituents have been found in nitrite and nitric oxide reductases from *Pseudomonas stutzeri* producing N_2 gas[18].

Evidence that Fe and Cu are constituents of the enzyme include (a) concentration of these metals in purified fractions of the enzyme, (b) activation of the enzyme in extracts of Cu and Fe deficient felts on returning the deficient metal. An external supply of Cu^{1+} will reduce nitrite non-enzymically; but the reduction is slightly enhanced in the presence of the enzyme and DPNH, presumably because Cu^{1+} is regenerated enzymically. Flavin, although it was not required for this reduction, was essential when DPNH was the donor. Whether or not Fe is a functional constituent of the enzyme remains to be established, since neither Fe^{2+} nor reduced cytochrome *c* acted as exogenous donors for the enzymic reduction of nitrite. The spectrum of purified fractions of the enzyme reduced with dithionite or by DPNH did not show any definite cytochrome bands, despite the fact that the enzyme was concentrated many fold by freeze drying.

The pyridoxine requirement for the enzyme confirms the results of SILVER AND MCELROY[4], who showed reduced nitrite reductase activity in felts of a pyridoxine requiring mutant of *Neurospora* and an accumulation of nitrite in the culture medium when the vitamin was deficient. In this paper it is shown that the enzyme is reconstituted quickly *in vivo* by returning pyridoxine to pyridoxine-deficient felts or by adding pyridoxine, pyridoxal, or pyridoxal phosphate to crude extracts of deficient felts. This *in vitro* effect was not, however, obtained with the purified enzyme and pyridoxal or pyridoxal phosphate did not concentrate in the enzyme, so that pyridoxine may not be directly involved in nitrite reductase action. One possible explanation is that, in the crude extract containing all the enzymes for nitrate assimilation, the addition of pyridoxine or pyridoxal formed an oxime chemically with hydroxylamine, thus removing one of the products of reduction, and thereby accelerating nitrite reductase activity. This would not occur in the purified enzyme since it was comparatively free from the other enzymes concerned in the reductive sequence. Silver and McElroy[4] have suggested that pyridoxine might operate in nitrite utilization by condensing free hydroxylamine with pyridoxal phosphate to form an oxime that is reduced to the amine and then by transamination reactions to amino acids regenerating pyridoxal phosphate. We have prepared a series of oximes by reacting various keto acids with hydroxylamine and we also used ^{14}C carboxyl-labelled pyruvic oxime; but we have no evidence that these are enzymically reduced to amino acids in *Neurospora*. The results show that the oxime formation is probably a method of detoxication and for storage of hydroxylamine prior to its enzymic reduction. The Mg requirement for the enzyme may also be an indirect effect, since it did not accumulate in purified fractions of the enzyme, nor did its addition to the purified enzyme activate it; but it is essential for enzyme formation.

The most purified fractions of the enzyme contained an active DPNH-cytochrome *c* reductase and DPNH-diaphorase but TPNH was without effect. Kinsky and McElroy[19] found that TPNH-cytochrome *c* reductase paralleled the fractionation of nitrate reductase from *Neurospora*; cytochrome *c* inhibited competitively nitrate reduction. In our experiments with nitrite reductase, however, neither cytochrome *c* nor 2.6 dichloroindophenol inhibited nitrite reduction. It is feasible that cytochrome *c* is acting here as would a suitable redox dye acceptor, so that cytochrome *c* reductase activities in both nitrate and nitrite reductases may not have physiological significance in the organism.

ACKNOWLEDGEMENTS

We are grateful to Mr. C. P. Lloyd-Jones, B.Sc., for providing a radiocounting service; to Mr. D. J. Fisher for assay of the flavin constituents and to Mr. G. L. Mabey for making the illustrations.

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GLUTAMINE SYNTHETASE IN ADULT-HEN-HEART TISSUE

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(Received April 11th, 1959)

SUMMARY

Cell-free extracts of adult hen-heart tissue have been shown to contain glutamine synthetase, which catalyses the synthesis of glutamine from glutamic acid. A partial purification of this enzyme has been achieved using ammonium sulphate and protamine sulphate fractionations. This enzyme has been found to require an optimum temperature of 50° and is inhibited by phosphate.

INTRODUCTION

Glutamine has been found by EHRENSVÄRD, FISCHER AND STJERNHOLM[1], WESTFALL, EVANS, PEPPERS, HAWKINS AND EARLE[2], and EAGLE[3] to be a necessary growth factor for mammalian cells in tissue culture. Chick-heart tissue seems to differ from mammalian cells in this respect: MORGAN AND MORTON[4] and GOTHOSKAR, RATNAM AND RAMAKRISHNAN[5] found that chick-heart tissue cultivated in a synthetic medium does not need glutamine for survival and growth. Since glutamine is known to be necessary for cellular nucleic acid synthesis, it is likely to be synthesised by chick-heart tissue from some precursor present in the synthetic medium used for its cultivation. The suggestion that glutamic acid may be such a precursor derives from the observation of DEMARS[6], who has shown that Hela cells grown in the medium of EAGLE *et al.*[7] supplemented with a high concentration of glutamic acid, develop increased amounts of glutamine synthetase, which is known to effect the conversion of glutamic acid into glutamine. This enzyme has been detected in sheep brain and pea seeds by ELLIOTT[8-10], in pigeon liver by SPECK[11], and in kidney by RICHTERICH VAN BAERLE, GOLDSTEIN AND DEARBORN[12]. This enzyme has recently been detected[13] in freshly prepared chick-heart tissue explants and has been found to show a maximal activity at the time of peak proliferation of the tissue when grown in a synthetic medium containing glutamic acid but no glutamine.

Attempts were therefore made to isolate this enzyme from fresh chick-heart tissue and study its characteristics. The results are reported in this paper.

MATERIALS AND METHODS

Isolation of glutamine synthetase

Three adult hens were killed by decapitation and their hearts removed. After removal of fatty portions and blood clots, the heart tissue was chopped to a fine pulp in a cold room at 0° and ground in a pestle and mortar for 20 min with 50 ml of 0.05 *M* sodium bicarbonate buffer, pH 7.4. The extract (A) was recovered by centrifugation at 18,400 × *g* and 0° for 30 min.

40 ml of the cell-free extract were brought to 20 % saturation with solid ammonium sulphate (14 g/100 ml) and allowed to stand at 0° for 10 min. The precipitate was removed by centrifugation at 4,600 × *g* and 0° for 10 min, and the supernatant was brought to 40 % saturation by adding a further 14 g of ammonium sulphate/100 ml of solution. The resulting precipitate was removed by centrifugation at 12,800 × *g* and 0° for 10 min, and dissolved in buffer to a final volume of 35 ml (B).

35 ml of fraction B were brought to 21 % saturation with solid ammonium sulphate (14.7 g ammonium sulphate/100 ml of fraction B taken) and allowed to stand at 0° for 10 min. The precipitate was then removed by centrifugation at 4,600 × *g* and 0° for 10 min and the supernatant brought to 35 % saturation by further adding 9.8 g of ammonium sulphate/each 100 ml of fraction B. The resulting precipitate was removed by centrifugation at 12,800 × *g* and 0° for 10 min and dissolved in buffer to a final volume of 28 ml (C).

To fraction C was added one volume of 2 % protamine sulphate solution. The inactive precipitate was removed by centrifugation at 4,600 × *g* and 0° for 10 min and the supernatant saved (D).

All the enzyme fractions were dialysed for one hour at 0° against 0.02 *M* bicarbonate buffer, pH 7.4, and tested for glutamine synthetase activity.

Materials

Tris(hydroxymethyl)aminomethane, (Sigma), Sodium ATP, (Sigma), L-glutamic acid, cysteine hydrochloride, and hydroxylamine hydrochloride were commercial preparations. Glutamic acid, cysteine hydrochloride, and hydroxylamine hydrochloride were neutralised with NaOH before use.

Methods

Glutamyl hydroxamic acid was estimated by the method described by ELLIOTT[14]. Protein in the crude enzyme preparation was estimated by the microkjeldahl method and that in other fractions by the method of WARBURG AND CHRISTIAN[15]. The circular paper chromatographic method of GIRI AND RAO[16] was used for qualitative detection of glutamine.

Enzyme assay

The assay system used was essentially the same as that of ELLIOTT[14] except that tris(hydroxymethyl)aminomethane buffer was used instead of glyoxaline–HCl buffer.

2.25 ml of the reaction mixture contained tris buffer (pH 7.6), 80 μM; Na-ATP, 12 μM; sodium-glutamate, 200 μM; $MgSO_4$, 40 μM; NH_2OH, 100 μM; cysteine, 30 μM; and 0.2 ml of enzyme preparation. A blank containing all the components with boiled enzyme instead of fresh enzyme was run side by side. After addition of the enzyme, the mixture was incubated for 1 h at 50°. After incubation, 0.75 ml of

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Abbreviations used: ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; CSH, cysteine hydrochloride; GHA, glutamyl hydroxamic acid; NH_2OH, hydroxylamine hydrochloride; ADP, adenosine diphosphate; PCMB, *p*-chloromercuribenzoate.

ferric chloride reagent (the reagent contained equal volumes of 10 % $FeCl_3 \cdot 6H_2O$ in 0.2 *N* HCl, 24 % TCA, and 50 % v/v HCl) was added. The protein was removed by centrifugation at 128 × *g* and the hydroxamic acid content of the supernatant estimated by measuring the absorption in Klett-Summerson photoelectric colorimeter using filter 54. Synthetic GHA was used as the standard.

One unit of the enzyme has been defined as the amount of enzyme which produces, under the standard test conditions, one µmole GHA. The specific activity is expressed as units/mg protein.

In experiments involving the detection of glutamine, the reaction mixture used was the same as that described above except that ammonium chloride was used instead of hydroxylamine. After incubation, the reaction was stopped by the addition of an equal volume of alcohol, the protein was removed by centrifugation at 128 × *g* and the supernatant was used for qualitative paper chromatographic detection of the glutamate utilised and glutamine formed.

RESULTS AND DISCUSSION

The purification of glutamine synthetase (Table I) resulted in a nearly 200-fold increase in specific activity in the protein of protamine sulphate supernatant. Attempts to purify the enzyme further by ammonium sulphate fractionation or by calcium phosphate gel adsorption were not successful. The purified preparation was found to be free from glutamyl transferase and adenosine triphosphatase. Overnight dialysis of the crude extract against bicarbonate buffer had no effect on enzyme activity, but dialysis of the protamine sulphate supernatant fraction inactivated the enzyme completely. The crude extract remained active for 15 days when stored at —10°, whereas protamine sulphate supernatant remained active for only 3 days.

TABLE I

PURIFICATION OF THE GLUTAMINE SYNTHETASE

Purification step	*Total volume (ml)*	*Total units*	*Protein (mg)*	*Specific activity (units/mg protein)*	*Recovery of total units %*
A. Crude extract	40.0	516	200*	2.58	100
B. 1st ammonium sulphate precipitation (0.2–0.4 saturation)	35.0	490	70	7.00	95
C. Second ammonium sulphate precipitation (0.21–0.35 saturation)	28.0	400	28	14.29	78
D. Protamine fractionation	56.0	336	0.56	600.00	66

* From 5.8 g wet weight of heart tissue.

Figs. 1–6 show the relationship of GHA formation to time and enzyme concentration, concentrations of glutamate and NH_2OH, pH, temperature, concentrations of ATP, CSH and $MgSO_4$, and concentrations of ammonium sulphate and phosphate added in the form of potassium phosphate buffer, pH 7.6.

It can be seen from the data obtained that the glutamine synthetase activity of chick-heart tissue is optimal at a pH of 7.6, and is, in this respect, similar to that isolated from green pea seeds[10]. However, the optimal temperature for its activity is

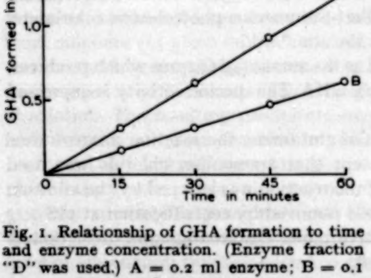

Fig. 1. Relationship of GHA formation to time and enzyme concentration. (Enzyme fraction "D" was used.) A = 0.2 ml enzyme; B = 0.1 ml enzyme.

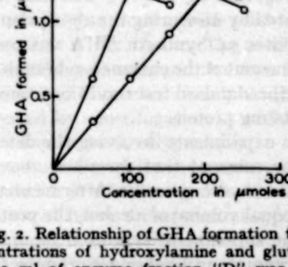

Fig. 2. Relationship of GHA formation to concentrations of hydroxylamine and glutamate (0.2 ml of enzyme fraction "D" was used). A = hydroxylamine; B = glutamate.

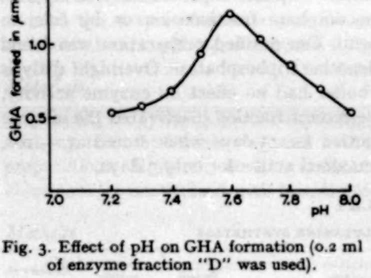

Fig. 3. Effect of pH on GHA formation (0.2 ml of enzyme fraction "D" was used).

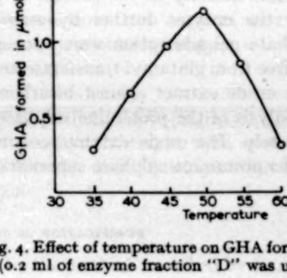

Fig. 4. Effect of temperature on GHA formation (0.2 ml of enzyme fraction "D" was used).

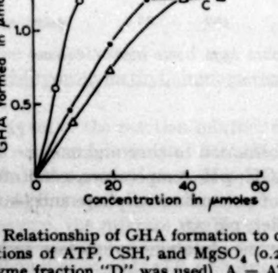

Fig. 5. Relationship of GHA formation to concentrations of ATP, CSH, and $MgSO_4$ (0.2 ml of enzyme fraction "D" was used). A = ATP; B = CSH; C = $MgSO_4$.

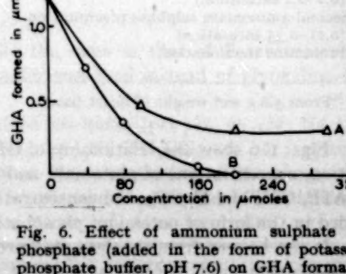

Fig. 6. Effect of ammonium sulphate and phosphate (added in the form of potassium phosphate buffer, pH 7.6) on GHA formation (0.2 ml of enzyme fraction "D" was used). A = ammonium sulphate; B = phosphate.

found to be 50°, whereas that for the enzyme isolated from other sources has been reported to be 30°. But in its inhibition by calcium, fluoride, ADP, and PCMB, and in its activation by magnesium, this enzyme seems to be a similar to that isolated from brain tissue[9]. Enzyme activity in the purified fraction was completely inhibited by 100 µg, of PCMB and could be fully restored by the addition of an extra 30 µmoles of cysteine. This shows the enzyme to be a sulphhydryl enzyme.

It is found that phosphate at a level of 200 µmoles completely inhibits enzyme activity and that ammonium sulphate at a level of 200 µmoles effects 73 % inhibition in the enzyme activity.

It was found that deletion of any component in the assay system except tris buffer resulted in an absence of GHA formation. In the absence of tris buffer, however, there was 75 % GHA formation.

It was confirmed by paper chromatographic studies that the end product formed was glutamine when NH_4Cl instead of NH_2OH was used in the assay system.

It is evident from the foregoing that glutamine synthetase is present in hen-heart tissue and that it can effect the synthesis of glutamine from glutamic acid. This may account for the non-requirement of glutamine by chick-heart tissue cultivated in a synthetic medium containing glutamic acid and ATP, but no glutamine.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to the Indian Council of Medical Research for sponsoring this research project, and to Sigma Chemical Company, U.S.A. for the valuable gift of ATP.

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CHANGES IN THE PHOSPHATASE ACTIVITY OF BAKER'S YEAST DURING THE GROWTH PHASE AND LOCATION OF THE PHOSPHATASES IN THE YEAST CELL

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(Received April 1st, 1959)

SUMMARY

Using phenolphthalein diphosphate as substrate, it was possible to demonstrate three peaks in the pH curve of the phosphatase activity of baker's yeast, one at the pH optimum of the acid phosphatase 4.3, and two on the alkaline side at pH 7.9 and 8.9. Mg^{++} ions inactivate the acid phosphatase by about 15 %, but double the activity of the alkaline phosphatase components. The brewer's bottom yeast was found to possess scarcely half of the acid phosphatase activity found in commercial baker's yeast.

The acid phosphatase of baker's yeast is located mainly on the cell surface; about one third, however, is within the cell. Both alkaline phosphatase components are located wholly inside the cell membrane. It was found that intact baker's yeast is able to hydrolyse fructose-1,6-diphosphate to fructose. Moreover, manometric tests confirmed that intact baker's yeast ferments fructose-1,6-diphosphate.

During the industrial cultivation of baker's yeast its nitrogen content decreases stage by stage, as does the phosphorus content during the cultivation of the last two stages. During the cultivation process the acid phosphatase activity first increases, then falls sharply to one tenth during the growth period of the precommercial A_4 stage when the cultivation conditions become more aerobic. The acid phosphatase activity, however, intensifies during the cultivation of the commercial yeast stage when the aeration increases and the phosphorus content of they east decreases still further. During this phosphorus starvation the activity of the acid phosphatase increases sharply, becoming even 8-fold in 8 h as compared with the seed yeast.

The changes in the activity of the alkaline phosphatase components during the cultivation of baker's yeast are smaller than those of acid phosphatase. The activity of the alkaline phosphatases up to the commercial yeast stage apparently follows the changes in the phosphorus content of the yeast cells. A sharp 4–6 fold increase in the activity is, however, observed during the experimental phosphorus starvation.

INTRODUCTION

The investigations of SCHÄFFNER AND KRUMEY[1] already give a fairly complete picture of the yeast phosphatases and their location in the yeast cell. According to these workers three different phosphatases are found in both baker's yeast and brewer's bottom yeast: acid phosphatase or "top yeast phosphatase", pH optimum 4, α-glycerophosphatase, pH optimum 6.4, and alkaline phosphatase, which has a pH optimum near 8. The acid and the alkaline phosphatases are unspecific, whereas the α-glycerophosphatase is rather specific. Mg^{++} ions inhibit the acid phosphatase[2,3] but activate both the α-glycerophosphatase and the alkaline phosphatase. Later, HOFFMANN-OSTENHOF and co-workers[4,5] reported that they had separated an alkaline phosphatase from both baker's yeast and brewer's bottom yeast, which has a pH optimum of about 9.1 and is activated by Mg^{++} ions.

In the baker's and brewer's yeasts studied by SCHÄFFNER AND KRUMEY[1] the acid phosphatase was found to be located on the cell surface, whereas the other phosphatase components, the α-glycerophosphatase and the alkaline phosphatase, act intracellularly. Intact baker's yeast thus hydrolyses the phosphoric esters studied only in an acid environment, the activity–pH curves being equal to those obtained with acid phosphatase. The observations of SPERBER AND RENVALL[6] and also the facts reported by WESTENBRINK *et al.*[7] support the view that the splitting off of thiamine pyrophosphate precedes the uptake of thiamine by intact baker's yeast. Later investigations by ROTHSTEIN AND MEIER[8,9] (*cf.* also[10]) using baker's yeast, have confirmed the opinion that the acid phosphatase is located on the cell surface. These authors, however, did not consider the possibility that a part of the enzyme might be located within the cell (*cf.*[10], p. 14).

Studying the enzymic changes of baker's yeast during the growth phase, we have this time chosen to examine the phosphatase activity. It can be assumed beforehand that the saccharase activity[11] and the decarboxylative activity[12] changed during the cultivation process owing to a decrease in the supply of nutrients or to a change of the growth conditions to a more aerobic character. Concerning the phosphatase activity of the yeast HOFFMANN-OSTENHOF[13] has mentioned, in addition to the fact that the commercial baker's yeast is poor in its content of acid phosphatase, that the mother yeast lots used by them were found to contain very variable amounts of acid phosphatase. He assumed that the cultivation conditions, such as the vigour of aeration, affect the content of acid phosphatase in the yeast. In the present investigation we have studied the changes in the activity of both the acid and the alkaline phosphatases.

EXPERIMENTAL

Materials and experimental procedure

The baker's yeast used was produced by the Rajamäki Factories of the State Alcohol Monopoly, Rajamäki. The brewer's yeast originated from the brewery of Oy Mallasjuoma, Lahti. The determinations were made from different stages of propagation of baker's yeast. The industrial cultivation process of baker's yeast and the treatment of yeast samples have been described previously[12]. The cultivation experiments on the laboratory scale were performed using 10 l lots in a thermostatic fermentometer constructed by Messrs. O. Dich (Denmark) equipped with an automatic feeding and aeration apparatus[14]. The industrial cultivation conditions were followed as closely as possible with respect to feeding, aeration and temperature. The nutrient solution was of the synthetic type. The amounts of saccharose, nitrogen, phosphorus and magnesium added were proportional to those used in the industrial cultivations; on the other hand, the trace elements and the growth factors were added according to the directions of WHITE[15]. In one batch the ammonium phosphate was replaced by ammonium sulfate. The nutrient solution was administered according to the "Zulauf" principle. The cultivation was continued for 8 h, at the end of which period the growth of the yeast was still very intense.

The dry yeast and freeze-thawed yeast preparations were prepared as previously described[16]. The octyl alcohol–glycerol cytolysate was prepared by adding 1 ml of glycerol and 2 ml of octyl alcohol to 20 g of yeast and mixing for about 15 min. The yeast was cytolysed with sodium chloride by rubbing with additional portions of solid salt.

The total nitrogen content of the yeast samples was determined with the micro-Kjeldahl method, and the phosphorus content according to the amidol method of Müller[17] as modified by Allen[18]. The phosphatase activity was determined according to Huggins and Talalay[19] using phenolphthalein diphosphate as a substrate. The substrate solution, containing no preservatives, was buffered to the desired pH using a veronal–acetate buffer. The reaction temperature was 37° and the time of reaction was 1 h. The reaction was interrupted by the addition of an alkaline buffer increasing the pH of the reacting solution to 10.5. The extinction of the solution, which turned red owing to the liberation of phenolphthalein, was determined at 540 mμ immediately after the interruption of the reaction, omitting the filtration or centrifugation of the mixture. A suspension to which the alkaline buffer had been added before the addition of the substrate was used for comparison. Since Mg^{++} ions activate the alkaline phosphatases, magnesium sulfate was added to the yeast suspension when the alkaline phosphatase activity was determined in order to make the final magnesium concentration in the reacting solution $0.8 \cdot 10^{-2}$ *M*.

In the determinations of the phosphatase activity in animal tissues Huggins and Talalay[19] found that the amount of phosphorus liberated from phenolphthalein diphosphate was directly proportional to the concentration of the enzyme, but the phenolphthalein liberated was a parabolic function of the enzyme concentration. In contrast to these results we found that the extinction of the phenolphthalein liberated is a direct function of the concentration of baker's yeast acid and alkaline phosphatase. This would mean that in our determinations of the phosphatase activity the reaction yielded only one, or perhaps two, products of hydrolysis in a constant proportion within a wide range of enzyme concentration, excluding only the smallest concentrations.

The unit for the phosphatase activity is chosen in such a way that 10 enzyme units in one hour at 37° liberate 1 mg of phenolphthalein from excess substrate. All experimental results are expressed per 100 mg dry matter.

RESULTS

The pH curve of the phosphatase activity and location of the phosphatases in the cell

Using dried baker's yeast of the semiaerobic A_3 stage with the addition of Mg salts, three distinct peaks can be noted on the phosphatase activity–pH curve, at about pH 4.3, 7.9 and 8.9 (Fig. 1). The pH optimum of one of the phosphatase components is equal to that of the previously known "alkaline phosphatase" of yeast, and the pH optimum of the other corresponds to that of the alkaline phosphatase isolated by Hoffmann-Ostenhof. According to Albers and Albers[2,3], Mg^{++} ions in a concentration of $8 \cdot 10^{-2}$ *M* inhibit the acid phosphatase (*cf.* Figs. 1 and 2). On the alkaline side, however, values twice as great were obtained with this magnesium concentration. No change in the pH optima could be found in the presence of Mg^{++} ions. Both components of the alkaline phosphatase were found to be thermostable: their activity did not show any decrease during the heating of a suspension of dried yeast at pH 6.0 for 30 min at 50°; on the contrary, a 20% increase in the activity was observed.

The activity of the acid phosphatase of dried baker's yeast cells was found to be of the same order of magnitude as that of intact cells (Figs. 1–3, Table II). On the other hand, with baker's yeast the freeze-thawed preparation (Table I) and the octyl alcohol–glycerol andso dium chloride cytolysates were found to be clearly more active than the intact cells. The acid phosphatase activity of intact yeast is only about 2/3 of the corresponding activity of a freeze-thawed preparation. This was also proved with phosphatase-rich A_3-stage yeast. A considerable part of the acid phosphatase is thus located inside the cell membrane. The alkaline phosphatase activity of intact baker's yeast can not be detected at all, not even in the presence of Mg^{++} ions (Figs. 2 and 3). The alkaline phosphatase components are thus located within the yeast cell beyond the reach of the substrate and the pH changes of the medium.

The pH curves of the phosphatase activities of intact and dried commercial baker's yeast and brewer's bottom yeast are shown in Fig. 3. The activity–pH curve

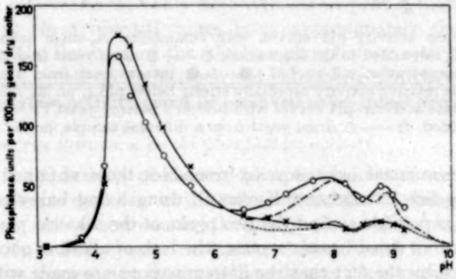

Fig. 1. Phosphatase activity–pH curves with dried A_3-stage baker's yeast (×——× no Mg salt added to the suspension, O——O $MgSO_4$ added, concentration $0.8 \cdot 10^{-2}$ *M*), and the alkaline part of the activity–pH curves with dried, phosphorus-starved baker's yeast (– – – – no Mg salt added, –·–·–·– $MgSO_4$ added, concentration $0.8 \cdot 10^{-2}$ *M*).

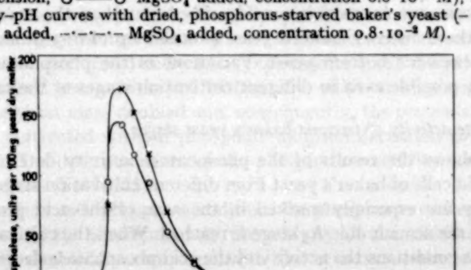

Fig. 2. Phosphatase activity–pH curves with intact A_3-stage baker's yeast (×——× no Mg salt added to the suspension, O——O $MgSO_4$ added, concentration $0.8 \cdot 10^{-2}$ *M*).

TABLE I

THE ACID PHOSPHATASE ACTIVITY OF INTACT BAKER'S YEAST CELLS COMPARED TO THAT OF VARIOUS YEAST PREPARATIONS

The determinations were performed using two samples from different cultivations of the commercial A_5-stage yeast. The results are expressed as phosphatase units per 100 mg yeast dry matter.

Sample No.	*Intact cells*		*Dried cells*		*Freeze-thawed cells*	
	pH 4.30	*pH 4.52*	*pH 4.30*	*pH 4.52*	*pH 4.30*	*pH 4.52*
1	36	33	—	—	55	53
2	39	38	44	39	59	55

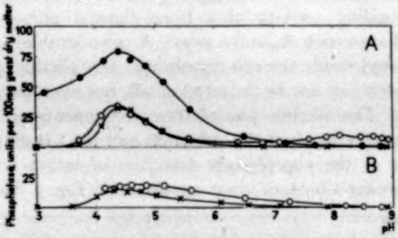

Fig. 3A. Phosphatase activity–pH curves with commercial A_5-stage baker's yeast (×——× intact yeast, no Mg salt added to the suspension, ○——○ dried yeast from the same cultivation, $MgSO_4$ added, concentration $0.8 \cdot 10^{-2}$ *M*, ●——● intact yeast from a different cultivation with the relative activity especially strong below pH 4, no Mg salt added).

Fig. 3B. Phosphatase activity–pH curves with brewer's bottom yeast (×——× intact yeast, no Mg salt added, ○——○ dried yeast from a different sample, no Mg salt added).

of the intact commercial baker's yeast resembles those obtained by ALBERS AND ALBERS[2] and by SCHÄFFNER AND KRUMEY[1], using living baker's yeast. With dry baker's yeast it is possible to find the two peaks of the alkaline phosphatase components. In the case of dried brewer's yeast, the lack of alkaline phosphatase activity may be explained by the fact that the determinations are made without addition of magnesium salt. The relative amount of acid phosphatase found in brewer's bottom yeast by ALBERS AND ALBERS[3] is considerably lower than the amount found by us. According to these authors, baker's yeast contains up to 8–9 times more acid phosphatase than brewer's bottom yeast. Variations in the phosphatase activity seem, however, to be possible even in different cultivation stages of the same yeast.

The phosphatase activity of various baker's yeast stages

Table II shows the results of the phosphatase activity determinations of both intact and dried cells of baker's yeast from different cultivation stages. The variations in the activity are especially marked in the case of the acid phosphatase, which increases until the semiaerobic A_3 stage is reached. When the cultivation is continued in more aerobic conditions the activity of the acid phosphatase decreases to one tenth during the growth of the precommercial A_4 stage. During the cultivation of the last stage, the commercial A_5 stage, the activity increases again, showing values up to four times those of the preceding stage.

The alkaline phosphatase activity of the dry yeast preparations obtained at

TABLE II

ACTIVITIES OF ACID AND ALKALINE PHOSPHATASES OF BAKER'S YEAST DURING THE INDUSTRIAL CULTIVATION PROCESS

The acid phosphatase activity has been determined without addition of $MgSO_4$, the alkaline phosphatase activity with $MgSO_4$ in a concentration of $0.8 \cdot 10^{-3}$ *M*. The results are expressed as phosphatase units per 100 mg yeast dry matter.

Yeast stage	*Average aeration* m^3/m^3 *soln./h*	*N content of yeast* (%)	*P content of yeast* (%)	*Acid phosphatase activity pH 4.3*		*Alkaline phosphatase activity dried yeast**	
				intact yeast (units)	*dried yeast (units)*	*pH 7.9 (units)*	*pH 8.9 (units)*
R_3	slight	9.50	2.11	112	136	57	53
A_2	1.3	8.12	2.08	166	168	34	32
A_3	34.4	7.71	2.28	171	164	41	33
A_4	38.6	7.46	1.87	17	17	25	20
A_5**	48.0	6.54	1.30	68	79	25	16

* The activities of alkaline phosphatases determined using intact yeast were in all samples analysed < 1 unit/100 mg dry matter.

** The phosphatase activity of A_5-stage yeast varied, being sometimes definitely smaller (see Table III, test series 2 and Fig. 4).

different stages of cultivation was only slightly lower at pH 8.9 than at pH 7.9, the relative activity at these two pH values being approximately the same for the different samples. The decrease of the alkaline phosphatase activity was roughly proportional to the decrease of the nitrogen and phosphorus content of the yeast cell. No alkaline phosphatase activity could be observed using intact yeast.

The effect of phosphorus starvation on the phosphatase activity

The changes in the activity of the acid phosphatase do not seem to follow the nitrogen and phosphorus depletion of the cells, but rather the change in cultivation conditions to a more aerobic state during the growth period of the A_4 stage. The acid phosphatase activity increases again during the cultivation of the A_5 stage, during which the conditions become even more aerobic, whereas the nitrogen and phosphorus content of the cells decreases. This observation appeared to agree well with the results of RAUTANEN[20,21] concerning an increase in the phosphatase activity of *Torulopsis utilis* during the phosphorus starvation. In order to demonstrate the effect of starvation of phosphorus we performed cultivation experiments on a laboratory scale using A_5-stage yeast as seed yeast, with and without the addition of phosphate. During the cultivation the yeast mass doubled and, consequently, the concentration of phosphorus in the lot cultivated without phosphate addition decreased to about half of the original value. The determinations of the phosphatase activity were made from dry yeast preparations (Table III).

In the cultivations with phosphate addition, the phosphorus content of the yeast increased by about 50%. Simultaneously the activity of the alkaline phosphatase was approximately doubled as compared with the activity of the seed yeast, but the activity of the acid phosphatase decreased 25–50%. During the phosphorus starvation, the activity of both the alkaline and the acid phosphatases increased sharply, the activity of the acid phosphatase increasing 3 to 8 times as compared with the activity of the seed yeast and 6 to 11 times as compared with the activity

TABLE III

CHANGES IN PHOSPHATASE ACTIVITY DURING PHOSPHORUS STARVATION OF COMMERCIAL BAKER'S YEAST

The samples have been taken from two different series of cultivation with and without phosphate addition. Phosphatase activity has been determined from dried yeast samples, the acid phosphatase activity at pH 4.3 without addition of $MgSO_4$, the alkaline phosphatase activity at pH 7.9 with $MgSO_4$ in a concentration of $0.8 \cdot 10^{-3}$ *M*. The results are expressed as phosphatase units per 100 mg yeast dry matter.

Time of cultivation (h)	*Test series No.*	*Without phosphorus added*			*With phosphorus added*		
		P content of yeast (%)	*Phosphatase activity*		*P content of yeast (%)*	*Phosphatase activity*	
			acid (units)	*alkaline (units)*		*acid (units)*	*alkaline (units)*
0	1	1.34	74	13	1.34	74	13
	2	1.41	42	9	1.41	42	9
5.5	1	0.80	236	50	2.01	42	18
	2	0.91	331	55	2.03	35	23
8	1	0.69	232	48	1.94	38	16
	2	0.69	340	44	1.81	32	27

of the yeast cultivated with addition of phosphate. The alkaline phosphatase activity increased 4 to 6 times as compared with the activity of the seed yeast, and 2 to 3 times as compared with the yeast cultivated with addition of phosphate.

The hydrolysis of hexose diphosphate

Since the location of the acid phosphatase is mainly on the cell surface, it is illogical to assume that the hexose diphosphate would be unfermentable by intact yeast, as has been claimed for decades[22–24]. This claim has been based on the fact that the intact yeast cell is impermeable to it (*cf.*[24,25]). However, ROTHSTEIN AND MEIER[9] have concluded, on the basis of the fact that intact yeast cells are impermeable to phosphorylated sugars, that glucose-6-phosphate and glucose-1-phosphate are fermentable by intact baker's yeast at a rate equal to the rate of dephosphorylation on the cell surface. Since it has been claimed that hexose diphosphate is easily hydrolysed by the acid phosphatase of yeast[1–3,26], we have re-examined the hydrolysis of hexose diphosphate with intact and dried baker's yeast. In order to obtain clear results the experiments on hydrolysis were performed using phosphorus-starved baker's yeast with high phosphatase activity. The experiments were carried out in 0.001 *M* fructose-1,6-diphosphate solutions buffered to the pH optimum 4.3 of acid phosphatase and containing 0.1 % yeast calculated as dry matter. The phosphate liberated was estimated according to the amidol method by ALLEN[18]. In 1 h at 37° the intact yeast hydrolysed 65.9 % of the phosphorus of fructose-1,6-diphosphate, the dried yeast preparation 65.2 %. The dried yeast showed with phenolphthalein diphosphate as substrate a phosphatase activity of 232 units and with fructose-1,6-diphosphate an activity of 243 units (10 units in this case being equal to 197 γ phosphorus liberated).

It can be seen that the hydrolysis of hexose diphosphate with both intact and dry yeast occurs at about the same speed as the hydrolysis of the phenolphthalein diphosphate. It was thus possible to liberate 2/3 of the phosphoric acid from the hexose diphosphate within one hour. This means that at least a part of the substrate had been completely hydrolysed to fructose—also by the action of the acid phosphatase located on the surface of the intact yeast cells. These results already show that intact yeast must be able to ferment fructose diphosphate.

The fermentation of fructose-1,6-diphosphate by intact yeast cells was then directly observed by manometric tests. It was found that the phosphorus-starved baker's yeast with high phosphatase activity fermented fructose-1,6-diphosphate about three times faster (330 μl CO_2/h/100 mg yeast dry matter) than the commercial A_5-stage baker's yeast (120 μl CO_2/h/100 mg yeast dry matter). This corresponds to the acid phosphatase activity ratio of the respective yeast (Test series 1 in Table III). The above results are thus in agreement with the experimental results obtained by ROTHSTEIN AND MEIER[9] with glucose monophosphates.

DISCUSSION

The acid phosphatase activity of baker's yeast increases during the industrial cultivation, as long as the growth conditions remain fairly anaerobic, but decreases sharply during the aerobic cultivation period of the precommercial A_4 stage. It is peculiar that in the vigorously aerated growth conditions of the commercial A_5 stage, the activity of the acid phosphatase again begins to increase, sometimes fairly strongly. This observation apparently tallies with the findings of RAUTANEN[20,21] that the phosphatase activity of *Torulopsis utilis* increases during phosphorus starvation. This was confirmed by the observation that a further increase of the acid phosphatase activity occurred when the A_5-stage yeast was cultivated experimentally in strongly aerobic conditions similar to those of the industrial cultivation process, but in a solution containing no phosphorus.

In the cultivation process of baker's yeast the activity of the acid phosphatase falls sharply during the precommercial A_4 stage, which is the first stage cultivated in completely aerobic conditions. The aeration as such is not, however, the decisive factor in the changes that occur in the activity of the phosphatase. This is seen by the increase of the activity during the cultivation of the commercial A_5 stage, and by the phosphorus starvation experiments with strong aeration in which a decrease was found in the acid phosphatase activity in the presence of phosphate, while in the absence of phosphate a sharp increase of the activity was evident. The observation by SPERBER AND RENVALL[6] that the activity of the acid phosphatase on the cell surface of baker's yeast showed no change during starvation of carbohydrate reserves through treatment shaking in oxygen atmosphere, is also in agreement with this view.

It seems as though the changes in the activity of the alkaline phosphatases would apparently follow the changes in the phosphorus content of the cells during the industrial cultivation of baker's yeast. During the phosphorus starvation of the commercial baker's yeast, a clear increase in the activity is, however, also observed on the alkaline side. RAUTANEN[20] found activity at pH 8.6 neither in normally cultivated nor in phosphorus-starved cells of *Torulopsis utilis* yeast, in contrast to the results of HOFFMANN-OSTENHOF[27], who found alkaline phosphatase activity.

Our experiments, which have been made using phenolphthalein diphosphate as substrate, naturally do not show the possible occurrence of α-glycerophosphatase in baker's yeast. However according to SCHÄFFNER AND KRUMEY[1] this enzyme is also found in baker's yeast.

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Biochim. Biophys. Acta, 37 (1960) 482–490

PROPRIÉTÉS DE LA L(+)-ALANINE-DÉSHYDROGÉNASE

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(Reçu le 17 avril, 1959)

SUMMARY

Properties of L(+)-alanine-dehydrogenase

An enzyme responsible for the reversible oxidative deamination of the L(+)-alanine is present in *Bacillus subtilis*. This enzyme, called L(+)-alanine-dehydrogenase, functions specifically with DPN.

The enzyme has been partially purified. It is relatively thermo- and alkali-stable, and is partially inhibited by sulfhydryl reagents. Its optimum pH is 10.

Homologues of alanine are very slightly oxidized by it; in order of decreasing activity they are: α-aminobutyric acid, serine, vinylalanine, valine and norvaline.

The energetics of the reaction and the Michaelis constants for the substrates have been determined. The specificity of the enzyme makes it suitable for use in the quantitative determination of L(+)-alanine in amounts of 0.1 to 0.3 μmoles.

Biochim. Biophys. Acta, 37 (1960) 490–502

INTRODUCTION

Il a été démontré précédemment[24] l'existence chez *Bacillus subtilis* d'un enzyme qui catalyse la désamination oxydative et réversible de l'alanine suivant l'équation (1):

$$CH_3\text{-}CH(NH_3^+)\text{-}COO^- + DPN^+ + H_2O \rightleftharpoons CH_3\text{-}CO\text{-}COO^- + DPNH + H^+ + NH_4^+ \quad (I)$$

L'intérêt de cette réaction est double. Elle peut réaliser une voie d'oxydation de l'alanine et elle pourrait également, en principe, correspondre à une voie d'entrée de l'ammonium dans les synthèses des amino-acides. Cette dernière fonction n'a été prouvée, jusqu'à présent, que pour la réaction catalysée par la déshydrogénase glutamique[1, 8, 23, 25]. La similitude entre cette dernière réaction et la désamination oxydative de l'alanine a conduit à appeler cet enzyme, "L(+)-alanine-déshydrogénase".

Seules les propriétés de l'enzyme, isolé d'une souche de *Bacillus subtilis*, seront présentees dans ce travail. L'étude de son rôle physiologique, ainsi que de sa distribution chez d'autres organismes, est actuellement en cours.

Nous avons développé ailleurs le problème qui a conduit à mettre en évidence l'alanine-déshydrogénase (AlDHase)[24]. Rappelons brièvement que dans des souches de *B. subtilis* dépourvues de déshydrogénase glutamique (souche Am⁻), l'acide glutamique est cependant oxydé. Nous avons été amenés à faire l'hypothèse que l'acide glutamique était oxydé via une transamination formant de l'alanine et cette dernière était alors déshydrogénée par l'AlDHase. Un nouvel argument est apparu qui renforce cette idée, le taux d'AlDHase s'accroît parallèlement au pouvoir de cette souche d'oxyder le glutamate.

Les propriétés de l'alanine-déshydrogénase qui sont présentées dans ce travail, concernent principalement sa spécificité et les relations de l'enzyme avec les substrats de la réaction. La purification quoique partielle, a été suffisante pour faire ressortir les propriétés d'intérêt biologique les plus importantes.

MATÉRIELS ET TECHNIQUES

Microorganismes et cultures

Nous avons utilisé un variant de *Bacillus subtilis*, la souche I.R.C. 6 (Am⁻S) obtenue dans notre laboratoire. Cette souche n'assimile pas l'ion ammonium, faute de déshydrogénase glutamique (Am⁻). L'azote assimilable doit être fourni sous forme d'acide glutamique ou d'un amino-acide qui peut se transformer en acide glutamique dans la cellule (arginine, ornithine et proline).

Pour la production maximum d'alanine-déshydrogénase, on a effectué les cultures dans le milieu suivant: glutamate de sodium (0.01 *M*); $(NH_4)_2SO_4$ (0.01 *M*); KH_2PO_4 et Na_2HPO_4, 0.02 *M* pour l'ensemble, de façon à réaliser le pH désiré, généralement pH 7.3; NaCl (0.01 *M*); K_2SO_4 (0.01 *M*); citrate de Fe ($0.4 \cdot 10^{-4}$ *M*); $MgSO_4$ (0.001 *M*).

Le sulfate de magnésium est ajouté après stérilisation du milieu. Il faut noter que dans ce milieu, l'acide glutamique est utilisé comme source d'azote et de carbone. Ceci triple approximativement la production d'alanine-déshydrogénase.

Pour les cultures allant de 40 ml à 2.5 l, les croissances ont toujours été réalisées dans des conditions d'aération provoquée par la rotation du liquide de culture. Le récipient est agité sur un plateau animé d'un mouvement de translation circulaire (120 à 150 tours/min — excentricité de 3 cm). Le rapport du volume du liquide à celui du récipient doit être tel que le mouvement du liquide dans le récipient soit circulaire. Par exemple, les cultures de 2.5 l sont effectuées en ballons de 6 l. Pour obtenir les bactéries en quantité adéquate à la production d'enzymes, nous avons effectué des cultures d'un volume de 30 l en ballons de 45 l. L'agitation, cette fois, était obtenue au moyen d'un axe muni de 6 pales s'ouvrant par rotation et tournant à une vitesse de 300 t/min, provoquant un cône d'air qui atteignait le fond du ballon. Dans ces conditions, les 30 l de milieu stérilisés à 100° sont ensemencés au moyen de 2 × 2.5 l de culture. La culture de 35 l n'est pas rigoureusement exempte de possibilité de contamination, mais ceci ne cause guère d'inconvénients vu la courte durée de la croissance (8 h). Les bactéries sont toujours recueillies au cours de la phase exponentielle de croissance (opacité de 0.6 à 0.7, mesurée à l'opacimètre Spekker et calculée pour 1 cm de chemin optique). On récolte 8 g (poids sec) de bactéries pour 30 l de culture. Ces bactéries son recueillies par centrifugation dans une centrifugeuse "Sharpless" et ensuite lavées à l'eau et lyophilisées. L'ensemble des essais a été réalisé au moyen de 50 g (poids sec) de bactéries.

Mesure des activités enzymatiques

Les activités de déshydrogénase sont suivies par la méthode classique, soit d'oxydation du DPNH ou de réduction du DPN$^+$. Alors que la mise en évidence qualitative de l'AlDHase est plus spécifique dans le sens de l'oxydation de l'alanine, le dosage de l'enzyme est effectué plus aisément par la réaction opposée en mesurant l'oxydation du DPNH. Le mélange réactionnel comprend: 0.05 ml de DPNH à 10 mg/ml; 0.15 ml de NH_4Cl, 3 M; 0.20 ml de pyruvate $M/6$ et 2.75 ml de triéthanolamine 0.05 M à pH 7.6. L'enzyme contenu dans un volume de 0.01 à 0.1 ml est ajouté en dernier lieu à l'aide d'un agitateur de forme spéciale[3]. La densité optique étant fixée arbitrairement au départ, on mesure le temps écoulé après l'addition de l'enzyme. Les lectures sont effectuées de 15 en 15 sec. et la concentration en enzyme doit être telle que les variations soient linéaires pendant au moins 45 sec.

L'unité d'alanine-déshydrogénase a été arbitrairement définie comme la quantité d'enzyme qui provoque une variation de densité optique (due à l'oxydation du DPNH) de 0.1 en 100 sec pour un volume de 1 ml et un chemin optique de 1 cm; les mesures étant effectuées à la longueur d'onde de 340 mμ, au spectrophotomètre Zeiss thermostatisé à 25°.

L'activité spécifique des préparations enzymatiques est exprimée en unités par mg de protéines. Celles-ci ont été déterminées par la méthode du biuret[21] ou par la méthode turbidimétrique de MEJBAUM[15].

Produits

Les acides aminés utilisés provenaient de la firme "F. Hoffmann-La Roche und Co.", sauf la vinyl-alanine (Sigma Chemical Co) et la L(—)-norvaline (Light and Co). Le DPN et le DPNH, purs, ainsi que le pyruvate de sodium, étaient fournis par "C.F. Boehringer und Soehne".

RÉSULTATS EXPÉRIMENTAUX

1. *Purification partielle de l'alanine-déshydrogénase*

Préparation des extraits de bactéries

(a) *Extraits bruts.* Les extraits ont été préparés par lyse au lysozyme. L'enzyme peut également être retrouvé dans les extraits obtenus au moyen d'ultra-sons (Raytheon 10 kc).

Dans le cas de la lyse au lysozyme, on a procédé comme suit: on incube 2 h à 30°, avec agitations fréquentes, un mélange de 5 g de bactéries lyophilisées, 0.1 g de lysozyme, 1 ml d'une solution de ribonucléase contenant 2.5 mg/ml, 1 ml de $MgSO_4$ 0.2 *M*, ces substances étant amenées à un volume final de 100 ml au moyen de triéthanolamine 0.05 *M* à pH 7.0. A la fin de la lyse, on ajoute 1 ml d'une solution à 0.01 % de désoxyribonucléase. L'action de la RNase facilite l'éloignement de l'acide ribonucléique, quant à la DNase elle liquéfie le gel fourni par la lyse et permet aussi d'éliminer plus aisément les éléments insolubles par centrifugation à $30,000 \times g$ pendant 60 min (Spinco, modèle L). Sauf indication, le reste des opérations est effectué à 0°.

(b) *Eloignement des acides nucléiques.* On ajoute lentement, sous agitation constante, une solution de $MnSO_4$ 1 *M*, à raison de 1/20 du volume de l'extrait[11]. Après 30 min, le précipité formé est éliminé par centrifugation. Le surnageant est dialysé 24 h contre du tampon triéthanolamine 0.01 *M* à pH 7.0.

(c) *Fractionnement au sulfate d'ammonium.* On opère par redissolution du précipité obtenu à saturation en sulfate d'ammonium aprés quelques heures; les résultats sont exprimés à la Fig. 1. Ces données nous ont conduit à retenir la fraction comprise entre 65 et 45 % de saturation. Cette mèthode accroît de 3 fois l'activité spécifique de la préparation.

(d) *Action de la température.* Des essais préliminaires ont montré que l'AlDHase était relativement thermostable. Un chauffage pendant 5 min à 65° ne détruit que 12 % de l'activité, alors que 75 % des protéines sont coagulées et éliminées par centrifugation. L'effet pratique est moindre, car une partie de l'enzyme est souvent adsorbée par les protéines dénaturées par la chaleur.

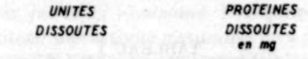

Fig. 1. Solubilité de l'alanine-déshydrogénase en fonction du degré de saturation en sulfate d'ammonium. L'extrait, précipité préalablement à 100 % de saturation au $(NH_4)_2SO_4$, est redissous progressivement par diminution de la saturation. Les courbes montrent la dissolution de l'alanine-déshydrogénase et de l'ensemble des protéines de l'extrait.

(e) *Adsorption sur gel de phosphate de calcium.* C'est principalement l'adsorption des protéines différentes de l'AlDHase qui a permis une purification ultérieure. Le gel de phosphate de calcium est préparé suivant KUNITZ[13], 0.5 mg (poids sec) de gel sont utilisés par mg de protéines dialysées. Après 30 min de contact, le gel est centrifugé. L'activité spécifique du mélange protéique double par cette opération. Des essais d'adsorption de l'enzyme sur le gel, qui peuvent être réalisés en portant le pH à 5.3, ne donnent guère d'avantages. Des phosphates 0.01 *M*, à pH croissant de 6 à 7.5, éluent l'ensemble des protéines parallèlement à l'AlDHase.

(f) *Diverses autres modalités de purification.* Les opérations décrites en (c), (d) et (e) furent employées pour préparer l'extrait enzymatique utilisé dans la suite de ce travail. Quelques autres modalités ont également été testées, toutefois, si elles peuvent être utiles, avec des extraits bruts, le résultat de leur action se superpose aux stades précédemment étudiés. Il s'agit, d'une part, de la résistance de l'enzyme aux pH alcalins. Une dialyse de l'extrait brut pendant 24 h contre du tampon borate à pH 10 double l'activité spécifique avec un rendement total de 80 %. Cette opération effectuée sur un extrait issu du stade (e), n'apporte pas de purification (voir Tableau I). D'autre part, l'électrophorèse de zone sur amidon qui a permis de purifier activement la β-galactosidase de *E. coli*[9, 19] ne donne dans le cas de l'AlDHase qu'une purification des extraits bruts; l'avantage disparaît avec un extrait partiellement purifié (stade e).

(g) *Exemple de purification.* Le Tableau I, réunit les données d'un exemple quantitatif de purification.

Des données du Tableau I il ressort que les stades de purification (b), (c), (d) et (e) seuls sont intéressants. On se bute aux difficultés habituelles de purification des enzymes bactériens dont les propriétés physico-chimiques voisinent avec celles de la majorité des protéines cellulaires. Limitée par la petite quantité de matériel initial, la purification est astreinte à éviter les pertes considérables de rendement. Comme il apparaîtra par la suite, le degré de purification obtenu est cependant suffisant pour étudier les propriétés d'intérêt biologique essentielles de l'enzyme. En particulier, l'enzyme ne manifeste pas d'activité de déshydrogénase lactique, de déshydrogénase glutamique et d'oxydase du DPNH. Toutefois, les caractéristiques qui ne peuvent être obtenues que par l'enzyme pur, telle que son activité spécifique molaire, ne peuvent pas être abordées.

TABLEAU I

STADES DE LA PURIFICATION DE L'ALANINE-DÉSHYDROGÉNASE

Opérations	*Activité totale en unités*	*Protéines totales en mg*	*Activité spécifique en unités par mg de prot.*	*Rendement en %*	*Purification obtenue*
Extrait initial centrifugé	1,550,000	1,950	795	100	0
Précipitation au $MnSO_4$	1,390,000	1,190	1,170	90	1.5 ×
1er fractionnement au $(NH_4)_2SO_4$ 65 — 45%	1,155,000	342	3,380	75	4.25 ×
Incubation à 65°	842,000	142	5,925	54	7.45 ×
Adsorption négative sur gel de $Ca_3(PO_4)_2$	792,000	119	6,650	51	8.40 ×
2e fractionnement au $(NH_4)_2SO_4$ 65 — 50%	607,000	93	6,530	39	8.20 ×
Dialyse contre tampon borate pH 10	457,000	73	6,260	30	7.90 ×

2. *Specificité de l'alanine-déshydrogénase*

La spécificité de l'alanine-déshydrogénase sera étudiée, d'une part, par la constante de dissociation de l'enzyme pour les différents substrats, ainsi que par la mesure de la vitesse maximum de la réaction. Dans ce dernier cas, faute de connaître le poids moléculaire de l'enzyme, on ne pourra pas donner de valeurs absolues, les valeurs seront exprimées comparativement à la vitesse de déshydrogénation de l'alanine.

(a) *Cinétique de la réaction en fonction des substances participant à la réaction* (*I*). On a recherché l'affinité des divers substrats de la réaction (I), par la méthode de Michaelis modifiée par LINEWEAVER ET BURK[14]. Le Tableau II résume les résultats de plusieurs expériences indépendantes.

TABLEAU II

CONSTANTES DE MICHAELIS DES DIFFÉRENTS SUBSTRATS ET COENZYMES DE LA L(+)-ALANINE-DÉSHYDROGÉNASE

Substrat	*Constante de Michaelis (tampon pyrophosphate pH 8 à 25°) M*	*Vitesse maxima (µM de coenzyme transformé en 1 min par unité d'AlDHase)*
L(+)-alanine	$3.6 \cdot 10^{-3}$	$4.3 \cdot 10^{-4}$
	$3.4 \cdot 10^{-3}$	$4.9 \cdot 10^{-4}$
DPN$^+$	$2.4 \cdot 10^{-4}$	$4.7 \cdot 10^{-4}$
	$2.9 \cdot 10^{-4}$	$5.2 \cdot 10^{-4}$
Ac. pyruvique	$8.8 \cdot 10^{-4}$	$1.5 \cdot 10^{-2}$
	$8.7 \cdot 10^{-4}$	$1.6 \cdot 10^{-2}$
DPNH	$3.7 \cdot 10^{-5}$	$1.7 \cdot 10^{-2}$
	$4.5 \cdot 10^{-5}$	$1.7 \cdot 10^{-2}$
	$4.2 \cdot 10^{-5}$	$1.3 \cdot 10^{-2}$
NH_4^+	0.37	$1.8 \cdot 10^{-2}$
	0.35	$1.8 \cdot 10^{-2}$

(b) *Amino-acides autres que la* L(+)-*alanine*. Plusieurs amino-acides montrent une faible activité. Pour obtenir une activité mesurable, on a dû augmenter fortement la concentration des substrats (de l'ordre de 500 à 1000 fois les concentrations utilisées pour la L(+)-alanine). Dans le cas de l'acide α-aminobutyrique, l'activité est suffisante pour exprimer la constante de Michaelis de la réaction. Pour les autres amino-acides, cette mesure était fort imprécise, c'est pourquoi on a uniquement mentionné la vitesse dans des conditions où l'enzyme est probablement saturé. Le Tableau III rassemble ces résultats.

Les données cinétiques montrent que l'enzyme est très spécifique de la L(+)-alanine. L'acide α-aminobutyrique qui, hormis l'alanine, est déshydrogéné le plus rapidement, a une constante de Michaelis 100 fois plus grande que la L(+)-alanine et, dans les conditions de saturation, ne manifeste qu'une vitesse égale à 13 % de celle de l'alanine. Il est donc certain que dans les conditions physiologiques, l'enzyme peut difficilement métaboliser l'acide α-aminobutyrique. Il est cependant intéressant de constater que l'acide α-aminobutyrique est l'homologue le plus proche de l'alanine. Les autres amino-acides qui montrent une certaine activité ont des structures qui

TABLEAU III

CONSTANTES DE MICHAELIS ET VITESSES MAXIMA DE L'ALANINE-DÉSHYDROGÉNASE AVEC DIFFÉRENTS ACIDES AMINÉS

Acide aminé	*Constante de Michaelis*	*Vitesse maxima*
L(+)-Alanine	$1.3 \cdot 10^{-3}$ *M*	100
Acide DL-α-aminobutyrique	$0.9 \cdot 10^{-1}$ *M*	13
DL-Sérine	—	1.5
Vinyl-alanine	—	1.0
L(+)-Valine	—	0.8
DL-Norvaline	—	0.8

Les vitesses maxima sont exprimées en % de la vitesse obtenue avec la L(+)-alanine. Conditions expérimentales: pH = 9.2 en tampon pyrophosphate et 25°.

s'écartent de l'alanine parallèlement à l'activité mesurée. Des amino-acides non naturels, comme la vinylalanine, peuvent être attaqués très lentement. Vu les fortes concentrations de ces amino-acides nécessaires pour la mise en évidence des activités, il n'est pas exclu que l'activité mesurée ne soit due à des impuretés. Cependant, l'allure de la cinétique, obtenue avec la sérine ou la valine, fait penser à une activité significative. Par contre, dans le cas de l'acide glutamique commercial, l'activité est caractérisée par une cinétique similaire à celle d'une faible quantité d'alanine. Dans ce cas, une impureté devait être suspectée, c'est ce qui fut confirmé par recristallisation de l'acide glutamique, dont on a pu, par cette méthode, montrer qu'il contenait de l'ordre de 1/1000 de L(+)-alanine. Ceci illustre une application analytique de cet enzyme.

L'éventuelle action de l'AlDHase sur la D(—)-alanine paraît le seul cas où une décision sur la nature de la faible activité observée, nécessiterait une purification parfaite de l'enzyme. En présence de très grandes quantités de D(—)-alanine, on observe une réduction lente du DPN$^+$. La cinétique de la réduction est donnée à la Fig. 2. L'allure de la courbe n'est pas celle de la L(+)-alanine en faible quantité, mais une complication apparaît du fait de l'inhibition de l'AlDHase par la D(—)-

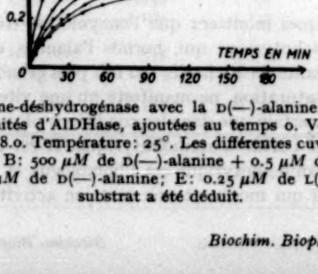

Fig. 2. Activité de l'alanine-déshydrogénase avec la D(—)-alanine. Chaque cuvette contient: 3.7 *μM* de DPN et 700 unités d'AlDHase, ajoutées au temps 0. Volume final: 3 ml. Tampon pyrophosphate: 0.1 *M*, pH 8.0. Température: 25°. Les différentes cuvettes contiennent en outre: A: 1 *μM* de L(+)-alanine; B: 500 *μM* de D(—)-alanine + 0.5 *μM* de L(+)-alanine; C: 0.5 *μM* de L(+)-alanine; D: 500 *μM* de D(—)-alanine; E: 0.25 *μM* de L(+)-alanine. Un blanc sans substrat a été déduit.

alanine, on ne peut donc pas exclure que la D(—)-alanine dont nous disposions, ne soit légèrement contaminée par la L(+)-alanine.

Vu l'allure des cinétiques, cette hypothèse est plus vraisemblable que celle d'une contamination par une racémase, enzyme qui est connu chez *Bacillus subtilis*[20]. De plus, la racémase de l'alanine requiert le pyridoxalphosphate, or, aucun effet accélérateur de la réduction du DPN^+ n'a été constaté par addition de ce dernier.

Plusieurs amino-acides ont été testés qui ne donnent, même à forte concentration, aucune réduction du DPN^+. Ce sont: la β-alanine, la glycine, la L(—)-phénylalanine, l'acide L(+)-aspartique, la L(+)-glutamine, la L(—)-leucine, la DL-norleucine et la L(+)-isoleucine.

L'action de l'AlDHase est spécifiquement liée au DPN, ainsi que l'a montré l'absence de toute activité en présence de TPN.

3. *Influence du pH*

La Fig. 3 montre que l'AlDHase a un optimum d'activité en milieu très alcalin. Le pH optimum n'a cependant pas pu être déterminé avec précision, faute d'un tampon efficace dans la région de pH 10, qui ne soit pas inhibiteur comme le sont les tampons borate–NaOH et glycine–NaOH. On s'est borné à porter le tampon pyrophosphate à différents pH jusque 10.2. Le pH optimum de 10 est à mettre en parallèle avec la grande stabilité de l'AlDHase en milieu alcalin, une propriété qui permet d'obtenir une purification partielle de l'enzyme.

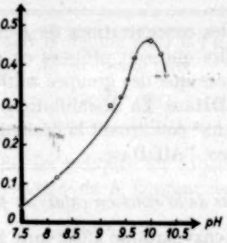

Fig. 3. Détermination du pH optimum d'activité de l'alanine-déshydrogénase. Pour chaque essai: 175 unités d'AlDHase, 50 μ*M* d'alanine, 1 μ*M* de DPN, tampon pyrophosphate: 0.1 *M*. Volume total: 3 ml. Température: 25°. Les pH sont mesurés en fin de réaction.

4. *Action des reactifs des groupes sulfhydryles*

On a étudié l'inhibition de l'activité de l'AlDHase par le *p*-chloromercuribenzoate (Fig. 4), l'*o*-iodosobenzoate et l'eau oxygénée.

L'*o*-iodosobenzoate ajouté aux concentrations de $0.5 \cdot 10^{-3}\,M$ et $1.0 \cdot 10^{-3}\,M$, n'exerce qu'une faible inhibition (15 %). Le *p*-chloromercuribenzoate aux concentrations de $1.5 \cdot 10^{-3}\,M$ et $3.0 \cdot 10^{-3}\,M$, produit des inhibitions qui sont respectivement de 26 et 37 %. Cette inhibition se manifeste également si le DPNH ou le pyruvate sont ajoutés avant l'inhibiteur. Des concentrations supérieures d'inhibiteur n'ont pas pu être réalisées. L'effet inhibiteur du *p*-chloromercuribenzoate est entièrement supprimé par un traitement ultérieur de l'enzyme par la cystéine (Fig. 4). L'eau oxygénée aux concentrations de $10^{-3}\,M$ et $2\cdot10^{-3}\,M$, en présence d'ions ferriques et cuivriques ($10^{-6}\,M$) et après 2 heures de contact, exerce une inhibition de 49 et 84 %. Quoique l'eau oxygénée ne soit pas un réactif entièrement spécifique des groupes sulfhydryles et que les concentrations de *p*-chloromercuribenzoate utilisées soient bien supérieures à celles qui sont utilisées en général dans ce genre d'essai, on est cependant porté à croire que des groupes sulfhydryles sont mis en jeu dans la catalyse produite par l'AlDHase. La désinhibition par la cystéine renforce cette opinion. Les résultats de BULEN[4] concernant la déshydrogénase glutamique sont fort semblables à ceux obtenus avec l'AlDHase.

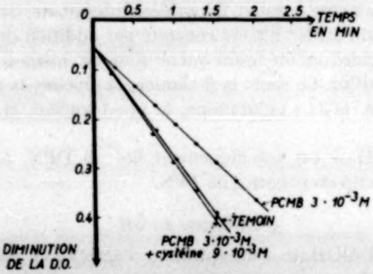

Fig. 4. Inhibition de l'activité de l'alanine-déshydrogénase par le PCMB et désinhibition par la cystéine. Mélange réactionnel: 0.2 ml de pyruvate $M/6$, 0.1 ml de DPN à 5 mg par ml, 0.1 ml de NH_4Cl 3 M et 17.5 unités d'AlDHase (soit 2.5 µg de protéines). Volume total: 3 ml. Tampon triéthanolamine 0.05 M, pH 8.0. Température: 25°. La cystéine, le pyruvate, le DPNH et l'NH_4^+ sont ajoutés après 10 min de contact de l'inhibiteur avec l'enzyme afin d'éviter tout effet de protection. Courbe 1: Témoin sans inhibiteur, ni cystéine; Courbe 2: Inhibiteur (concentration finale de $3\cdot10^{-3}\,M$); Courbe 3: Inhibiteur (concentration finale de $3\cdot10^{-3}\,M$) + cystéine (concentration finale de $9\cdot10^{-3}\,M$).

5. *Energetique de la reaction catalysée par l'AlDHase*

En l'absence d'une réaction enzymatique effectuant la transformation de l'alanine en pyruvate, BURTON ET KREBS[5,6,12] ont déterminé l'énergie libre de la réaction (II) en combinant plusieurs réactions enzymatiques connues.

$$\text{Alanine}(\pm) + H_2O \rightleftharpoons \text{Pyruvate}(^-) + NH_4(^+) + H_2 \qquad \text{(II)}$$

Ils ont ainsi établi que l'énergie libre standard de cette réaction est de $\Delta G^\circ = 13{,}120$ calories. Au moyen de l'alanine-déshydrogénase, on peut actuellement mesurer directement cette valeur. On a mesuré la constante d'équilibre donnée par l'équation:

$$K = \frac{(\text{Pyruvate}^-)\,(\text{DPNH})\,(NH_4^+)\,(H^+)}{(\text{Alanine}\pm)\,(\text{DPN}^+)\,(H_2O)}$$

La Fig. 5 exprime les cinétiques conduisant aux équilibres à divers pH, elles sont dirigées dans le sens: alanine ⟶ pyruvate. Les valeurs de (DPNH) peuvent être données[10] sur la base d'une extinction molaire de $6.22\cdot10^{-6}$. La concentration initiale en DPN^+ est mesurée par sa réduction enzymatique au moyen d'AlDHase, en présence

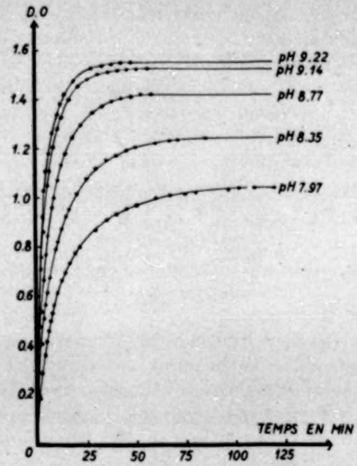

Fig. 5. Influence du pH sur l'équilibre de la réaction catalysée par l'alanine-déshydrogénase. Le mélange réactionnel comporte: 20 μM d'alanine, 0.79 μM de DPN, 1400 unités d'AlDHase dans un volume total de 3 ml. Tampon pyrophosphate: 0.1 M. Température: 25°. Pour chaque pH, un blanc sans substrat a été déduit.

d'un grand excès d'alanine (50 μM d'alanine pour 0.3 μM de DPN). En convenant de prendre pour (H_2O) la valeur 1, K est donné par:

$$K = \frac{(\mathrm{DPNH})^3\,(\mathrm{H}^+)}{(\mathrm{Alanine})\,(\mathrm{DPN}^+)}$$

La mesure des concentrations finales indiquée au Tableau IV, ainsi qu'une seconde série de mesures ont donné les valeurs de K figurant au Tableau III. La valeur de

TABLEAU IV

DÉTERMINATION DE LA CONSTANTE D'ÉQUILIBRE DE LA RÉACTION CATALYSÉE PAR L'ALANINE-DÉSHYDROGÉNASE

pH	*(H^+) × 10^{10}*	*Variation de d.o.*	*(DPNH) × 10^3*	*(Alanine) × 10^3*	*(DPN) × 10^3*	*K × 10^{14}*
* 7.97	107	0.841	0.135	3.20	0.128	6.42 ± 0.56
7.98	105	1.048	0.168	6.50	0.095	8.03 ± 0.74
* 8.32	47.8	1.073	0.173	3.16	0.091	8.60 ± 0.81
8.35	44.7	1.246	0.200	6.47	0.063	8.74 ± 0.93
* 8.73	18.6	1.297	0.209	3.12	0.055	9.88 ± 1.03
8.77	17.0	1.420	0.228	6.44	0.035	8.85 ± 1.07
* 9.10	7.94	1.431	0.230	3.10	0.034	9.30 ± 1.13
9.14	7.25	1.520	0.244	6.42	0.019	8.49 ± 1.38
9.22	6.03	1.546	0.249	6.42	0.015	9.67 ± 2.03
* 9.41	3.24	1.530	0.246	3.09	0.018	8.83 ± 1.55

Concentrations initiales: Alanine: $6.67 \cdot 10^{-3}$ M ou $3.33 \cdot 10^{-3}$ M (*), DPN $0.264 \cdot 10^{-3}$ M, tampon pyrophosphate 0.1 M. Volume total: 3 ml. Température: 25°.

Au temps 0, addition de 1400 unités d'AlDHase.

la précision exprimée est basée sur une erreur possible de 0.5 % dans les mesures de densité optique, des concentrations initiales d'alanine et de DPN, ainsi que sur une erreur absolue de 0.02 unité sur le pH. Sauf une seule valeur, la constante d'équilibre ne varie pas avec le pH en dehors des limites de variation imputables à la précision des mesures. La valeur moyenne de la constante d'équilibre est de $8.96 \cdot 10^{-14}$ *M*, ce qui correspond à une variation d'énergie libre standard $\Delta G^\circ = 17{,}790$ calories; à pH 7, $\Delta G' = 8{,}240$ calories — pour la réaction (I). Si on adapte la valeur de $\Delta G' = -4{,}330$ pour la réaction étudiée par BURTON ET WILSON[7]:

$$\mathrm{DPN^+ + H_2 \rightleftharpoons DPNH + H^+},$$

on trouve un $\Delta G'$ (pH 7) de $8{,}240 + 4{,}330 = 12{,}570$ calories pour la réaction (II), page 498. Cette valeur peut être comparée à celle de 13,120 obtenue indirectement par BURTON ET KREBS.

Il faut remarquer que dans les conditions physiologiques, la réaction procède en faveur de l'amination réductive du pyruvate; cependant, jusqu'à présent, seule la fonction physiologique de désamination a été démontrée. (Toutefois BRAUNSTEIN[25] et coll. ont apporté recemment des arguments fondamentaux qui indiquent que l'AlDHase peut être effectivement la voie d'assimilation de l'ammonium chez des *Bacillus*.)

6. *Utilisation de l'AlDHase pour le dosage de l'alanine*

La spécificité de l'AlDHase pour la L(+)-alanine permet de prévoir que cet enzyme rendra possible le dosage de cet amino-acide pour lequel on ne possède pas de méthode spécifique très sensible. La très faible réactivité de quelques amino-acides, comme l'acide α-aminobutyrique ou encore, à un degré moindre, la sérine, ne peut pratiquement pas intervenir. La D(—)-alanine ne peut également pas influencer les résultats dans le cas où l'enzyme est purifié comme il est décrit dans ce travail. En procédant en présence de 0.1 à 0.3 μM d'alanine et de 5 μM de DPN$^+$ à pH 9.6 en tampon pyrophosphate 0.1 *M*, on obtient une excellente proportionalité. La constante d'équilibre permet de calculer que le DPNH apparu correspond à 98.4 % de l'alanine mise en jeu dans le cas où 0.3 μM d'alanine est présente. Dans des mélanges biologiques contenant des ions ammonium, il faut procéder à l'élimination de ceux-ci par un chauffage préalable à pH alcalin.

DISCUSSION

Les propriétés de l'alanine-déshydrogénase décrites dans ce travail, ont une analogie frappante avec la déshydrogénase glutamique. Non seulement la structure de la réaction est similaire, mais on retrouve cette analogie dans de nombreux autres détails. En particulier, les affinités de l'enzyme pour les substrats et le coenzyme sont fort semblables (Tableau V). L'affinité pour l'ammonium est toujours faible, elle est d'ailleurs la plus faible dans le cas de l'AlDHase. La déshydrogénase glutamique est très spécifique de l'acide glutamique, toutefois, elle réagit avec le cétovalérate[2]. Alors que la déshydrogénase des tissus animaux réagit avec les coferments TPN et DPN, celle des micro-organismes est liée spécifiquement au TPN. L'AlDHase de *Bacillus subtilis* est spécifique du DPN.

TABLEAU V

COMPARAISON DE LA DÉTERMINATION DES CONSTANTES DE MICHAELIS DE L'ALANINE-DÉSHYDROGÉNASE ET DE LA DÉSHYDROGÉNASE GLUTAMIQUE

Substrats et coenzymes	*Constantes de Michaelis*				
	Déshydrogénase glutamique				*AlDHase (ce travail)*
	STRECKER	OLSON ET ANFINSEN	BULEN	BÄSSLER *et coll.*	
Glutamate ou alanine	$1.9 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$	—	—	$3.5 \cdot 10^{-3}$
DPN⁺	$2.5 \cdot 10^{-5}$	$1.0 \cdot 10^{-4}$	—	—	$2.6 \cdot 10^{-4}$
α-Cétoglutarate ou pyruvate	$1.2 \cdot 10^{-4}$	$7.0 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$	—	$8.8 \cdot 10^{-4}$
DPNH	$1.8 \cdot 10^{-5}$	$9.6 \cdot 10^{-5}$	$3.7 \cdot 10^{-5}$	—	$4.1 \cdot 10^{-5}$
α-Cétovalérate	—	—	—	$6.6 \cdot 10^{-3}$	—
α-Aminobutyrate	—	—	—	—	$0.9 \cdot 10^{-1}$
Autres amino-acides	—	—	—	—	10^{-1}
NH_4^+	0.057	0.056	0.101	—	0.36

La sensibilité aux réactifs des groupes sulfhydryles de la déshydrogénase glutamique du foie de boeuf étudiée par OLSON ET ANFINSEN[18] et celle des feuilles de blé étudiée par BULEN[4] est fort semblable à celle de l'AlDHase.

Malgré leur similitude d'action, alors que la déshydrogénase glutamique est certainement la voie générale d'amination en α, y compris chez les bactéries[23], aucun indice n'existe jusqu'à présent d'une fonction similaire pour l'AlDHase et une réponse à ce sujet nécessitera des travaux ultérieurs[25].

Une activité d'oxydation réversible, liée au DPN, de plusieurs aminoacides, comprenant l'alanine, a été montrée chez des *Clostridia* par NISMAN ET MAGER[16, 17]. Ce mécanisme enzymatique, qui n'a pas été complètement élucidé, apparaît différent de l'AlDHase, à la fois par sa spécificité et son exigence en phosphate.

REMERCIEMENTS

Ce travail a été subventionné par l'I.R.S.I.A. (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture). L'un d'entre nous (A.P.) remercie cette Institution pour l'octroi d'une bourse d'études. Nous remercions également la firme "F. Hoffmann-La Roche et Cie" pour le don de L(—)-sérine et de D(—)-alanine.

RÉSUMÉ

Il existe chez *Bacillus subtilis* un enzyme qui catalyse la désamination oxydative et réversible de la L(+)-alanine. Son action est couplée spécifiquement à la réduction du coferment I. L'enzyme a été appelé L(+)-*alanine-déshydrogénase.*

L'enzyme se classe dans les protéines constituant la masse la plus importante des protéines cellulaires, mais il a pu être purifié en tirant parti de quelques propriétés qui lui sont propres, en particulier, sa résistance à la chaleur et aux alcalis. Son pH optimum est voisin de 10. Cet enzyme est partiellement sensible aux inhibiteurs des groupes sulfhydryles.

La L(+)-alanine-déshydrogénase catalyse la déshydrogénation de la L(+)-alanine

à une vitesse beaucoup plus grande que pour tout autre acide aminé. Les quelques acides aminés homologues qui montrent une légère activité sont par ordre décroissant d'activité: l'acide α-aminobutyrique, la sérine, la vinylalanine, la valine et la norvaline. Une faible activité observée vis-à-vis de la D(—)-alanine est néanmoins douteuse.

Les constantes de Michaelis de la L(+)-alanine, du pyruvate, du DPN, du DPNH et de l'AlDHase ont été déterminées.

La spécificité de l'enzyme permet son utilisation pour le dosage de la L(+)-alanine en très faible quantité (0.1 à 0.3 μM).

L'étude de l'équilibre de la réaction a permis de déterminer directement l'énergétique de celle-ci.

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THE INDUCED SYNTHESIS OF CATALASE IN *RHODOPSEUDOMONAS SPHEROIDES*

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(Received April 10th, 1959)

SUMMARY

Synthesis of catalase is induced in *Rps. spheroides* on contact with air. In cultures grown anaerobically in the light the catalase content is 0.002 % of the dry wt. In aerobic cultures grown in darkness the catalase content is about 0.15 %. The rate of induced synthesis of catalase is low in a culture growing exponentially and high in a culture whose growth is limited through depletion of carbon sources. The slow induced synthesis in the former case is not accelerated by adding casein hydrolyzate. Rapid induced synthesis is maintained in a growing culture if glutamate and acetate are omitted from the growth medium.

Conditions of gratuity prevail for this induced synthesis of catalase if the culture is aerated gently in the light; rates of growth and induced synthesis are then independent of prior aeration and of catalase content. The rate of aerobic growth in darkness is also independent of catalase content. The kinetics of induction are similar for a culture aerated gently in the light (photosynthetic growth) and a culture aerated vigorously in darkness (aerobic growth). Synthesis of catalase is not related, directly or inversely, to synthesis of bacteriochlorophyll.

The induced synthesis of catalase requires sources of carbon, nitrogen, and energy. These requirement and the effects of inhibitors suggest that the protein of catalase is synthesized *de novo*. Although aeration causes this induced synthesis, energy for the process can be derived either from air or light.

INTRODUCTION

Our understanding of the biosynthesis of proteins has been advanced by the study of inducible enzymes[1]. An inducible enzyme that possesses a prosthetic group (*e.g.*, a hemoprotein) could be useful in studying the mechanism by which prosthetic group and protein are joined. In cases where the physiological function of an enzyme is uncertain, the function might be elucidated in an organism that can be made to contain either a high or a low concentration of the enzyme. The phenomenon of excitability, traditionally studied by sensory physiologists, is exhibited when an organism responds to a change in its environment by making an enzyme. Induced enzyme synthesis is also a special example of cellular differentiation. Thus a variety of problems can be broached through the study of induced enzyme synthesis.

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** Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

With these prospects in mind, an induced synthesis of catalase in the facultative photoheterotroph *Rhodopseudomonas spheroides* is being investigated. This organism can grow either aerobically in the dark or anaerobically by photosynthesis; oxygen is not liberated in its photosynthetic metabolism. The induced synthesis of catalase occurs on the introduction of oxygen. This paper describes some aspects of the kinetics, energetics, and chemistry of this induced synthesis.

In the study of induced enzyme synthesis, and especially in the interpretation of the kinetics of induction, a considerable clarification is achieved if "conditions of gratuity", as defined by MONOD AND COHN[2], are made to prevail[1]. Such conditions are said to prevail if neither the presence of the enzyme nor its inducer influences the general cellular metabolism. In studying the induced synthesis of β-galactosidase in *Escherichia coli*, MONOD AND COHN[2] achieved gratuity by using methyl-β-D-thiogalactoside as the inducer. This substance is not a substrate for metabolism, and under the conditions of growth, β-galactosidase was not involved in the general metabolism of the cells. Conditions of gratuity were attained by CHANTRENNE AND COURTOIS[3], in the study of induced catalase synthesis in yeast, by using a mutant that lacks respiratory enzymes (here the inducer is air). For the induced synthesis of catalase in *Rps. spheroides* the attainment of gratuity under illumination with gentle aeration will be demonstrated.

METHODS

Rps. spheroides, strain 2.4.1, was cultivated in a synthetic medium containing minerals, chelating agents, growth factors, malate, glutamate, and acetate, as described by COHEN-BAZIRE *et al.*[4]. Photosynthetic cultures were grown anaerobically under illumination at 30° in glass-stoppered bottles; illumination was provided by three 40-W tungsten lamps at a distance of about 12 in. Aerobic cultures were grown in darkness at 25° in agitated vessels.

Catalase was assayed by the iodometric method of HERBERT[5]. Intact cells were exposed to toluene for 4 min immediately before assay to render them freely permeable to H_2O_2. Samples that could not be assayed at once were treated with 0.2 μg/ml of tetracycline and placed in darkness; this treatment prevented further changes in their catalase content. Nonenzymic and peroxidatic decomposition of H_2O_2 in the assay were ruled out through observation of the reaction kinetics, oxygen evolution, and the effects of azide and heating. The catalase of *Rps. spheroides* has been purified and characterized[6]. A knowledge of its specific activity permits the expression of catalase content as percentage of the dry wt. From its molecular weight, the number of catalase molecules per bacterium can be estimated.

Growth was measured turbidimetrically, at 680 mμ, by the method of COHEN-BAZIRE *et al.*[4].

Catalase content and growth cycle

In a mature culture of *Rps. spheroides* grown anaerobically in the light (henceforth abbreviated Lt. Anaer.), the catalase content is about 0.002 % of the dry wt. If this culture is used as an inoculum for a culture grown aerobically in darkness, growth and catalase synthesis proceed as shown in Fig. 1 where the data from 4 experiments are superimposed. In one experiment covering the range 0 to 5 h the inoculum was 10%; here the data for cell mass and catalase content were divided by 100 to correspond to a 0.1% inoculum. In the other 3 experiments the inoculum was 0.1%.

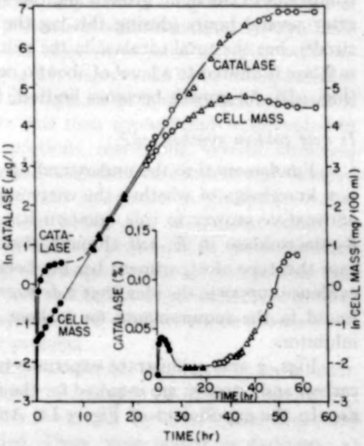

Fig. 1. Growth and catalase synthesis in *Rps. spheroides* growing aerobically in darkness at 25°. Inoculum: 0.1% (v/v) of a mature culture grown anaerobically in the light. In the inset the ordinate is catalase content (percentage of the dry wt.). Data of 4 similar experiments are represented by ●, ▲, ○, and △.

Fig. 1 shows that, in a culture growing aerobically in darkness from an inoculum of Lt. Anaer. culture, an initial outburst of catalase synthesis is followed by a slackening, after which the synthesis of catalase keeps pace with growth. As growth becomes limited[*], the synthesis of catalase continues for several hours.

The inverse experiment is shown in Figs. 2A and B. Fig. 2A represents a culture growing exponentially in the dark with aeration, which was transferred to anaerobic

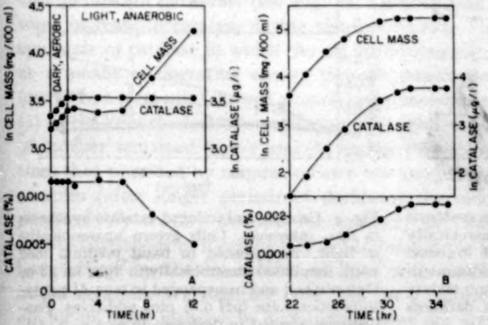

Fig. 2. A. Growth and catalase synthesis in *Rps. spheroides* transferred at t = 1.5 h from aerobic conditions (dark, 25°) to anaerobic conditions (light, 30°). B. Growth and catalase synthesis in *Rps. spheroides* growing anaerobically in the light at 30°. Inoculum: 0.1% (v/v) of a mature culture grown aerobically in darkness.

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[*] In all of the experiments to be described, growth in the complete medium became limited through an exhaustion of sources of carbon. This was ascertained by measuring final cell yield as a function of the initial concentrations of malate, acetate, and glutamate.

conditions in the light (in replicate glass-stoppered bottles) and allowed to grow to completion. Fig 2B portrays the development of a Lt. Anaer. culture from a 0.1 % inoculum of a culture grown aerobically in darkness. Fig. 2A reveals that, when *Rps. spheroides* is transferred from aerobic conditions in darkness to anaerobic conditions in the light, growth and the synthesis of catalase cease. Growth is resumed after several hours (during this lag the photosynthetic pigments are being synthesized[4]), but the total catalase in the culture remains constant. Eventually, when the catalase is diluted to a level of about 0.001 %, the synthesis of this enzyme is resumed (Fig. 2B). As growth becomes limited, the catalase content rises to about 0.002 %.

Is new protein synthesized?

Fundamental to the understanding of any example of induced enzyme synthesis is a knowledge of whether the enzyme-protein is synthesized *de novo*. A conclusive affirmative answer to this question has been obtained for the induced synthesis of β-galactosidase in *E. coli* through studies of radioisotope incorporation[7,8]. In our case this type of experiment has not been performed, but considerable circumstantial evidence supports the idea that a *de novo* synthesis of protein occurs. This evidence is found in the requirements for carbon, nitrogen, and energy and in the action of inhibitors.

Figs. 3 and 4 illustrate experiments showing that nonproteinaceous sources of carbon and nitrogen are required for the induced synthesis of catalase in *Rps. spheroides*. In the experiment of Fig. 3 Lt. Anaer, cells were washed and resuspended, in

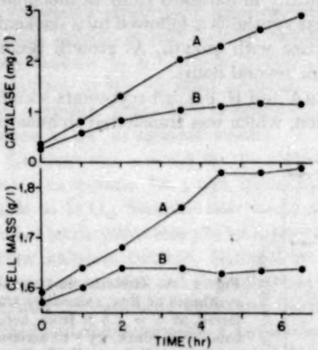

Fig. 3. Growth and induced catalase synthesis in *Rps. spheroides*. Cells grown anaerobically in light, washed, and resuspended in basal medium (see text) containing 0.02 *M* ammonium malate (curves A) or 0.02 *M* sodium malate (curves B). Suspensions aerated in darkness from t = 0.

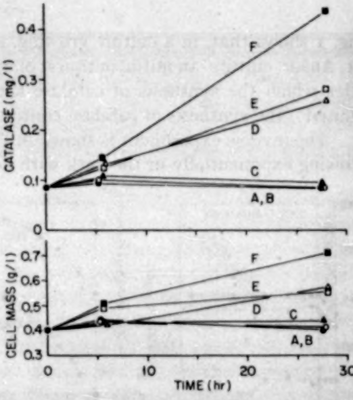

Fig. 4. Growth and induced catalase synthesis in *Rps. spheroides*. Cells grown anaerobically in light, resuspended in basal medium (see text), incubated anaerobically in light for 48 h, then washed and resuspended in 0.02 *M* potassium phosphate (pH 6.8) plus additives. Suspensions aerated in darkness from t = 0. Additives: curve A, none; curve B, $(NH_4)_2SO_4$; curve C, Na malate; curve D, NH_4 malate; curve E, Na acetate + Na-H glutamate; and curve F, NH_4 malate + Na acetate + Na-H glutamate. All additives were present initially at a concentration of 0.02 *M*.

2 aliquots, in "basal medium" plus 0.02 *M* ammonium malate (aliquot A) or 0.02 *M* sodium malate (aliquot B). The basal medium was the same as the complete growth medium except that it lacked ammonium ion, nitrate, malate, acetate, and glutamate. The resuspended aliquots were aerated in darkness. Growth and catalase content were measured at intervals. In the presence of ammonium malate, a vigorous synthesis of catalase proceeded for more than 6 h. In the presence of sodium malate, this synthesis ceased after 4 h, probably because endogenous reserves of fixed nitrogen were exhausted. In the experiment illustrated in Fig. 4 Lt. Anaer. cells were resuspended in basal medium and incubated anaerobically in the light for 48 h, to exhaust endogenous reserves of carbon and nitrogen. The culture was then washed and resuspended in 0.02 *M* potassium phosphate, pH 6.8. Six portions, containing various additives (see Fig. 4), were aerated in darkness and growth and catalase content were assayed at intervals. No catalase was synthesized by cells suspended in phosphate buffer (curve A), nor by cells suspended in phosphate buffer plus ammonium sulfate or sodium malate (curves B and C). Synthesis of catalase proceeded in the presence of phosphate plus ammonium malate (curve D), or phosphate plus sodium acetate plus monosodium glutamate (curve E). Rates of induced catalase synthesis were proportional to growth rates. Thus the induced synthesis of catalase requires sources of carbon and nitrogen other than intracellular proteins.

The roles of light and air in making energy available, and of air as an inducer, were demonstrated in the following experiment. A mature Lt. Anaer. culture was diluted 2.5-fold with complete medium, shaken with air for 1 min, and dispensed into glass-stoppered bottles, completely filled. These were placed in darkness; at intervals one was opened, and its contents were assayed for catalase. Other bottles were placed in the light after various intervals of darkness, and after 30-min illumination their contents were assayed. One bottle was opened after 130 min in darkness. The contents were shaken with air for 1 min, returned to the bottle, illuminated for 30 min, and assayed. Two bottles, one illuminated and the other in darkness from the start of the experiment, contained 0.001 % methylene blue. The dye was bleached, signalling the exhaustion of dissolved oxygen, after 110 min (illuminated) and 30 min (in darkness) (see Fig. 5). Samples kept in darkness (curve D) showed some increase in catalase during the first 20 min. This is interpreted as an induced synthesis of catalase in which the air introduced initially served both as inducer and as a means of liberating energy through respiration. The first illuminated sample (curve L, 0 to 30 min) showed a much larger increase in catalase content, for 2 reasons: (a) In the light the reduction of dissolved oxygen was slower, so that the concentration of inducer remained higher and (b) energy provided by photosynthesis was greater than that provided by respiration at a low oxygen tension. In successive illuminated samples (after longer periods of darkness) the induced synthesis of catalase was progressively smaller because the inducer, air, was being exhausted. The slight induced synthesis in a completely anaerobic sample (curve L, 135 to 165 min) might have arisen by the interaction of light with oxidizing entities generated by the initial aeration. We shall see later that, in a culture that has had no contact with air for 24 h, an increase in illumination does not act as an inducer. Curve A, L represents the sample that was aerated again after 130 min and then illuminated. Induced synthesis of catalase in this sample was nearly as great as that in the first illuminated sample; therefore inducibility was not lost on standing in darkness without air. These results show that the induced synthesis of catalase in *Rps. spheroides* requires energy made available by light or air, but air (not light) acts as inducer.

Several inhibitors were found to exert parallel effects on growth and on the induced synthesis of catalase. These results strengthen the likelihood that the latter process involves a synthesis of new protein.

Thus chloramphenicol caused a 50 % inhibition both of growth and catalase synthesis at a concentration of 1 μg/ml and complete inhibition of both processes at 10 μg/ml. For tetracycline the corresponding figures are 0.01 and 0.1 μg/ml.

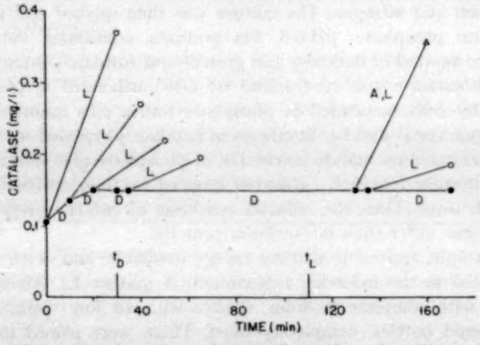

Fig. 5. Induced catalase synthesis in *Rps. spheroides* illustrating roles of light and air. Cells grown anaerobically in light, diluted 2.5-fold with complete medium, aerated 1 min, and dispensed into glass-stoppered bottles at t = 0. Curve D, bottles kept in dark. Curves L, bottles in dark until start of curve, then in light. Curve A, L, bottle in dark until start of curve, then contents aerated 1 min, returned to bottle, and illuminated. Dissolved O_2 exhausted in darkened bottle at time t_D; in illuminated bottle at time t_L.

After irradiation with u.v. an inhibition of growth and catalase synthesis occurred that became progressively more severe. A dose that depressed the rate of growth to 50 % of normal 4 h after irradiation reduced the rate of induced catalase synthesis to 60 % of normal.

In dim light (below saturation for photosynthesis) the rates of growth and catalase synthesis were depressed to the same extent. At one intensity both rates were 30 % of maximal, at another, 70 %.

A plot of growth rate versus pH had the same shape as a plot of rate of induced catalase synthesis versus pH. For both cases the rate was maximal at pH 7.1.

Kinetics of induction: gratuity; feedback mechanisms

The kinetics of the induced synthesis of catalase in *Rps. spheroides* appear to be dominated by the physiological state of the organisms. This is illustrated in Fig. 6, where the numbers on the curves represent approximate values for molecules of new catalase/cell/min. The data of curves A, B, and C were obtained with suspensions that were illuminated and aerated very gently (about 5 bubbles/min through 50 ml of suspension in a large test tube). Curve A shows the rapid, sustained induced synthesis of catalase, initiated and maintained by aeration in a Lt. Anaer. culture, whose growth had become limited by a depletion of carbon sources (other types of limitation have not been investigated). If such a culture is diluted with complete medium at the start of aeration (curve B), the rate of induced synthesis falls dramatically as growth is resumed. A Lt. Anaer. culture in exponential growth shows, when aerated, a slow but fairly constant rate of induced synthesis (curve C). In this case the differential rate of synthesis (new catalase/new cell mass) is nearly constant. Curve D shows the constitutive synthesis of catalase in an illuminated anaerobic culture in exponential growth.

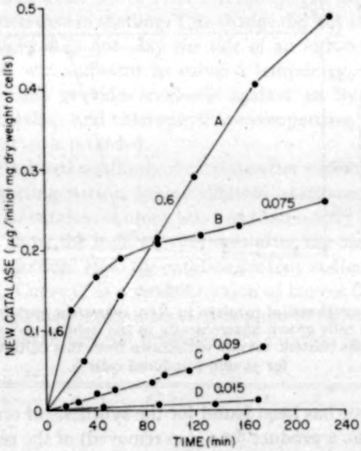

Fig. 6. Induced synthesis of catalase in *Rps. spheroides* in different stages of growth. Curve A, culture grown anaerobically in light to maturity (growth limited by exhaustion of carbon) then aerated gently in light from t = 0. Curve B, same as A, but culture diluted fivefold with complete medium at start of aeration. Growth began without lag. Curve C, illuminated, anaerobic culture in exponential growth, aerated in light from t = 0. Curve D, illuminated, anaerobic culture in exponential growth, showing the constitutive synthesis of catalase in the absence of air. The ordinate is normalized to an initial cell density (at t = 0) of 1 mg (dry wt.)/ml. Actual initial and final cell densities in mg/ml were 2.1 and 2.1 (A), 0.42 and 0.71 (B), 0.33 and 0.51 (C), and 0.63 and 0.95 (D). The numbers adjacent to the curves are approximate values for molecules of new catalase/cell/min.

The greater inducibility of cells in a carbon-limited culture is shown again in Fig. 7. Curves A and B show the data of Fig. 2B, which represent anaerobic growth and catalase synthesis in the light. Curve C shows the catalase content of cells withdrawn from this culture and aerated in the light for 30 min ("induced cells"). The inducibility rises sharply as growth is limited.

An increased rate of induced enzyme synthesis in "stationary-phase" cells has been reported for many systems[9, 10]. In some cases[9] the slow induction in exponentially growing cells is augmented by adding amino acids. In our case no such augmentation could be demonstrated by adding casein hydrolyzate or yeast extract. On the contrary, the inducibility remains high in a growing culture if glutamate and acetate are omitted from the medium, which ordinarily contains glutamate, acetate, and ammonium malate. An interpretation is that a specific organic substance, or group of substances, represses induction in a growing culture and that this substance is destroyed as growth becomes limited through exhaustion of certain carbon sources.

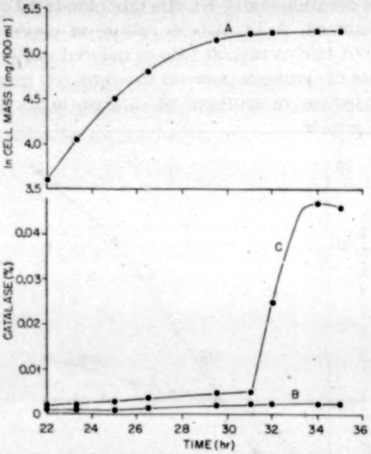

Fig. 7. Rate of induced synthesis of catalase in *Rps. spheroides* versus growth phase. Curves A and B represent data on cells grown anaerobically in the light at 30° and assayed immediately. Curve C shows the catalase content of cells withdrawn from this culture and aerated in the light for 30 min ("induced cells").

Such a specific inhibitor has been found for the synthesis of ornithine transcarbamylase in *E. coli*. Arginine, a product (one step removed) of the reaction of this enzyme, represses its synthesis[11]. Through this feedback mechanism the syntheses of citrulline and arginine can be regulated. In the present case, repressors could be the products of peroxidatic reactions involving catalase. Their identification might yield evidence concerning the natural substrates of catalase, acting peroxidatically.

The kinetics of induction might be modified further in this system by the presence or absence of conditions of gratuity. Gratuity can be defined, for our purposes, as follows. The action of oxygen as an inducer of catalase synthesis in *Rps. spheroides* is not modified by other effects of aeration such as a shift from photosynthetic to oxidative metabolism, nor does the newly formed catalase influence the subsequent induction. One can hope that this definition of gratuity was satisfied under the combination of illumination and gentle aeration employed in the experiments of Fig. 6. When a suspension aerated in this way was placed in a glass-stoppered bottle with methylene blue and illuminated, the dye was not bleached for 2 to 4 h. Respiratory utilization of oxygen was therefore slight; the energy for anabolic activities was provided mainly through photosynthesis. It is possible, however, that this degree of aeration was inhibitory to photosynthesis. It is also possible that catalase influences its own induced synthesis through a feedback mechanism: a rise in catalase content could alter the steady-state concentrations of H_2O_2 and of the catalase–H_2O_2 complex under constant aeration. CHANTRENNE described evidence for such a mechanism in yeast[12]. Finally, the rate of photosynthesis in the presence of air could depend on the catalase content. These questions were explored in the experiment illustrated in Fig. 8. A mature Lt. Anaer. culture of *Rps. spheroides* was diluted fivefold with complete medium under strict anaerobiosis. An aliquot was aerated gently in the light and growth and catalase content were assayed at 30-min intervals (curve A). The remainder was allowed to grow anaerobically in the light; samples were withdrawn at 30-min intervals. These were assayed immediately (curve B) or after 30 min gentle aeration in the light (curves C).

Curve B shows the constitutive synthesis of catalase in an anaerobic culture. The initial dilution of the culture led to a marked increase in the average illumination of the cells, owing to a decrease in shading. This change did not stimulate the synthesis of catalase, therefore light does not play the role of an inducer in this system. The increase in illumination was sufficient to cause a temporary cessation of bacteriochlorophyll synthesis[4]; this provides evidence against an hypothesis[13] that holds that the syntheses of catalase and chlorophyll are competitive, so that the former is accelerated when the latter is retarded.

Curves C show the induced synthesis of catalase after various periods of anaerobic growth in the light, starting with a freshly diluted "stationary" culture. For each of these curves the initial catalase content was less than 0.0025 %. The corresponding increments of curve A show the induced synthesis after the same interval of photosynthetic growth with aeration. Here the catalase content at the start of an increment was as high as 0.018 %. Curve C′ is a reconstruction of curves C for comparison with curve A. We see that each increment of curve A is equal to the corresponding C curve. Moreover, the rate of growth was not influenced by aeration (upper curve in Fig. 8).

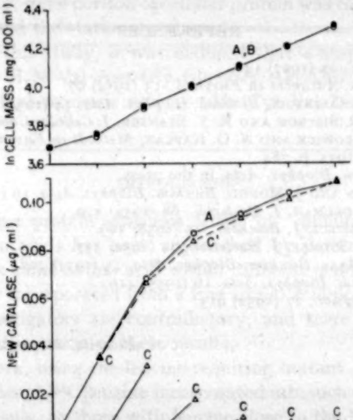

Fig. 8. Effect of prior aeration on the induced synthesis of catalase in *Rps. spheroides*. Cells grown anaerobically in the light to maturity, then diluted fivefold with complete medium, under strict anaerobiosis, at t = 0. A, aliquot aerated in the light from t = 0. B, aliquot illuminated without aeration from t = 0. C, samples illuminated without aeration from t = 0, then aerated in the light for 30 min. Curve C′ is constructed by joining curves C together.

Thus a prior history of aeration influences neither the rate of growth nor the rate of induced catalase synthesis, within the limits of this experiment. More specifically, rates of growth and induction are independent of catalase content for values of the latter ranging from 0.0025 to 0.018 %. In another experiment, which gave the same results, the catalase content ranged from 0.0026 to 0.045 %.

To summarize the results of this experiment: (a) Conditions of gratuity as defined in this section were satisfied. (b) The induced synthesis of catalase is not related to a cessation of bacteriochlorophyll synthesis. (c) A feedback mechanism involving catalase and H_2O_2 does not operate under the conditions of this experiment.

Conditions of gratuity provide a firm basis for the interpretation of data concerning induced enzyme synthesis[1]; it is for this reason that this point has been emphasized. It appears, however, that the induction of catalase synthesis in *Rps. spheroides* follows the same pattern whether gratuity prevails or not. Thus the kinetics of induction under vigorous aeration in darkness are similar to those under gentle aeration in light. Also, the rate of aerobic growth in darkness does not depend appreciably on the catalase content: when a Lt. Anaer. culture is transferred to aerobiosis in the dark, growth usually continues at the same rate without a lag. These facts emphasize our ignorance of the function of catalase in this organism.

ACKNOWLEDGEMENTS

This work was performed during the tenure of a National Science Foundation Senior Postdoctoral Fellowship.

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STUDIES ON THE MECHANISM OF PROTEIN SYNTHESIS

NONUNIFORM INCORPORATION OF [^{14}C]LEUCINE INTO α-AMYLASE AND THE PRESENCE OF α-AMYLASE PRECURSOR

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(Received April 29th, 1959)

SUMMARY

Using the leucine-requiring mutant of *Bacillus subtilis* and the α-amylase system, the incorporation of [^{14}C]leucine into cellular protein and such various leucine residues of the α-amylase molecule, as those with leucine close to the C-terminal amino acid, close to the N-terminal amino acid and in eight other positions on the protein molecule was estimated. "Unequal" labelling along a peptide chain, which suggested a "sequential" synthesis of protein, was demonstrated. Leucine residues in cellular protein, which did not increase so much after an addition of [^{14}C]leucine, were much more incorporated by [^{14}C]leucine than those of α-amylase, the greater part of which was produced during this period. Accordingly, some portion of cellular protein was converted into α-amylase after cell growth reached the stationary phase.

In the course of this study, it was deduced that α-amylase is a single chain polypeptide which has H_2N–Val·Asp·Gly·Glu·(Ser·Ala·Val)·(Leu·Ileu·Threo·Gly·Lys)··· as a N-terminal sequence and ···(Ala·Tyr·Val·Leu)·Asp(NH_2).OH as a C-terminal sequence.

INTRODUCTION

The problem of whether protein synthesis occurs by "simultaneous" or "stepwise" condensation of amino acids has been approached by determining whether or not various residues of the same amino acid within different peptide fractions of several proteins are uniformly incorporated from a free amino acid pool[1–8]. The observations made by several investigators are contradictory, and there have been many discussions[4,9] on the interpretation of these results.

In the present work, using the leucine-requiring mutant of *Bacillus subtilis*, the relative specific activities of [^{14}C]leucine incorporated into such various leucine residues of the α-amylase molecule, as those with leucine close to the N-terminal amino acid, close to the C-terminal amino acid and in eight other positions on the protein molecule, were estimated. "Unequal" labelling along a peptide chain, which is more readily interpretable in terms of a "sequential" synthesis of protein, was demonstrated. It was also observed that some portion of cellular protein, which may be considered as an α-amylase precursor, was excreted into the culture medium as an active α-amylase after the cell growth had reached the stationary phase.

MATERIALS AND METHODS

Bacterial strain

The leucine-requiring mutant of *Bacillus subtilis* strain K was used in these experiments. This mutant was obtained from the wild-type parent by M. KOHIYAMA at the Institute of Applied Microbiology of the University of Tokyo.

Materials

Carboxypeptidase-A prepared from beef pancreas[10] was used after recrystallizing 9 times. DL-[1-^{14}C]leucine (0.012 mC/mg) was purchased from Daiichi Pure Chemicals Co. Ltd. (Japan). Other materials used were the same as described in a previous paper[11].

Culture method

The leucine-requiring mutant was grown on the surface of the synthetic basal medium[11] supplemented with 180 mg/l of L-leucine.

Isolation and crystallization of α-amylase

α-Amylase was prepared from the culture medium by the procedure described previously[11].

Separation of N-terminal peptide from α-amylase

DNP-derivative of α-amylase was hydrolysed by trypsin at 25° for 20 h under stirring. The substrate was present in a concentration of 0.5 % in 0.1 *M* phosphate buffer at pH 7.8, and the concentration of trypsin (50 % $MgSO_4$) was 0.005 %. The reaction mixture was acidified with 6 *N* HCl to below pH 2, and washed with ether and ethyl acetate until the washings were colorless. Then the residue was extracted with methyl ethyl ketone until the extracts were colorless. The methyl ethyl ketone extract was washed with small amount of water and evaporated to dryness. The separation of the DNP-derivative of an N-terminal peptide from the evaporated sample was effected on a 10 × 0.9 cm column of Hyflosupercel buffered with 0.2 *M* phosphate buffer at pH 6.3, using methyl ethyl ketone saturated with the same buffer as an eluting solvent.

Hydrolysis of denatured α-amylase with carboxypeptidase

Acid-denatured α-amylase was hydrolysed by carboxypeptidase at 25° and pH 8. The substrate was present in a concentration of 0.5 % and the concentration of carboxypeptidase treated with diisopropylfluorophosphate was 0.02 %. After the reaction mixture had been treated with Amberlite IR-120, the amino acids liberated were estimated by quantitative paper chromatography[12] using Whatman No. 4 and *tert*-amyl alcohol saturated with water, and Whatman No. 4 and *n*-butanol–formic acid–water (75:15:10).

Determination of C-terminal amino acid of α-amylase by hydrazinolysis

The catalytic hydrazinolysis method devised by KAWANISHI *et al.*[13] was adopted. Hydrazinolysis was carried out for 12, 24 and 36 h. The DNP-derivative of C-terminal amino acid hydrazide liberated was estimated by paper chromatography[14].

Separation of peptide from various portions of α-amylase

A hydrolysate obtained after exposing acid-denatured α-amylase to the action of trypsin for 20 h was chromatographed on a column of Dowex-50-X2[11]. Each fraction was dried and converted to the dinitrophenyl derivative in the usual way; the reaction mixture was washed with ether, acidified with 6 *N* HCl and again washed with ether. The residual solution was extracted with methyl ethyl ketone until the extracts were colorless. The extract was washed with a small amount of water and evaporated to dryness.

Estimation of relative specific activity of [^{14}C]leucine

Each of the DNP-derivatives of peptides and α-amylase was hydrolyzed completely in 6 *N* HCl at 110° for 24–28 h. In the case of cellular protein, acetone-dry cells were washed twice with 10 % trichloracetic acid containing L-leucine (2 mg/ml), and again washed three times with 10 % trichloracetic acid. The residue was washed with ethanol and evaporated to dryness. The dry powder was hydrolysed as described above.

For separation of leucine from an acid hydrolysate, "double screening" by paper chromatography was adopted. After developing a hydrolysate and a standard solution of leucine, using Whatman No. 4 and *sec*-butanol–formic acid–water (15:3:2), the leucine spots were cut out and eluted[15]. An evaporated sample which contained leucine and (or) isoleucine was developed, using Whatman No. 4 and *tert*-amylalcohol saturated with water. Leucine spots were cut out, eluted and transferred to cups for measuring the radioactivity. Leucine location was marked using as a guide a spot of standard leucine made visible with ninhydrin.

In the case of the culture medium, it was deproteinized, desalted with ion-exchange resin[16] and treated as described above.

Radioactivity was measured using a windowless gas-flow counter. The amount of leucine was estimated by the ninhydrin color method after measuring the radioactivity. The measurements were performed with a number of different amounts of the same sample ranging from 0.03 to 0.15 μmoles of leucine, and the counts actually observed generally ranged from a hundred to a few thousand counts/min. The overall experimental error was not greater than ± 10 %.

Assay of α-amylase activity

The enzyme activity was measured by the method previously described[11] α-Amylase activity was represented as Wohlgemuth's unit ($D^{30'}_{40°}$)[17].

RESULTS

N-terminal amino acid sequence of α-amylase

A complete hydrolysate of the DNP-derivative of α-amylase was developed by two-dimensional paper chromatography[14]; only valine was found as an N-terminal amino acid. A methyl ethyl ketone extract obtained from a tryptic hydrolysate of the DNP-derivative of α-amylase was separated into three fractions, *viz.* a main fraction $R_F = 1$, a small fraction $R_F = 0.3$ and a trace fraction $R_F < 0.1$, by column chromatography on Hyflosupercel. The yield of the main fraction from 1 mole of α-amylase was more than 0.6 mole. The composition of the DNP-peptide expressed in terms of the molar ratios of the constituent amino acids is as follows: DNP-valine (1), aspartic acid (1), glycine (2), glutamic acid (1), serine (1), alanine (1), valine (1), leucine (1), isoleucine (1), threonine (1) and ε-DNP-lysine (1).

It has been reported that the N-terminal amino acid sequence of α-amylase prepared from the culture medium of *Bacillus subtilis* strain N is H_2N–Val·Asp·Gly·Glu·(Ser·Ala·Val)···[18]. Assuming that α-amylase produced by strain K has the same N-terminal amino acid sequence as that of strain N, the N-terminal sequence may be considered as H_2N–Val·Asp·Gly·Glu·(Ser·Ala·Val)·(Leu·Ileu·Threo·Gly·Lys)···. The position of one of the leucine residues may be between the 8th and 12th position from the N-terminal amino acid of α-amylase.

C-terminal amino acid sequence of α-amylase

By the hydrazinolysis method, only di-DNP-aspartic acid-β-hydrazide was detected by paper chromatography. The yield of di-DNP-aspartic acid-β-hydrazide obtained from 1 mole of the protein was about 0.9 mole, after hydrolysis for various periods ranging from 12 to 36 h.

By the carboxypeptidase method, after hydrolysis for 5 min, 0.4 mole of asparagine, 0.3 mole of leucine, 0.2 mole of valine, 0.2 mole of tyrosine and 0.1 mole of alanine were liberated from 1 mole of the protein. After further hydrolysis for several hours, 0.9 mole of leucine, 1.2 moles of tyrosine, 0.5 mole of valine and 0.8 mole of alanine, were liberated (asparagine was not measured). From these results, it may be concluded that the C-terminal amino acid sequence of α-amylase is ···(Ala·Tyr·Val·Leu)·Asp(NH_2).OH. One of the leucine residues must be in the position close to the C-terminal amino acid.

Relationship between bacterial growth and α-amylase production

The courses of bacterial growth and α-amylase production in the culture medium are indicated by the curves shown in Fig. 1. It will be seen that bacterial growth reached almost the stationary phase within a period of 50 h, and that the α-amylase activity as well as an amount of soluble protein in the medium then increased abruptly.

Incorporation of ^{14}C-leucine into α-amylase and cellular protein

In view of the results shown in Fig. 1, after an incubation of 50 h, 6 mg/l of DL-[1-^{14}C]leucine (0.012 mC/mg) was added to the culture medium; and after further incubation for 24 h, α-amylase was prepared from the medium.

The relative specific radio activities of leucine isolated from the culture medium, cellular protein and various portions of the α-amylase molecule are given in Table I. It was shown that the relative specific activity of [^{14}C]leucine in cellular protein (14,000 counts/min/μmole) was much higher than that in α-amylase molecule (4,600 counts/min/μmole). As is shown in Fig. 1, the greater part of the α-amylase accumulating in the medium was formed after addition of [^{14}C]leucine; on the other hand, cell growth was not so remarkable during this period. These results, together with the fact that little α-amylase could be detected within the cells*, show that some portion of cellular protein is converted to α-amylase and excreted after cell growth has

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* This observation[19], confirmed by the present authors, excludes the possibility that the increase of α-amylase in the medium depends upon a release of the enzyme from dying cells. Moreover the α-amylase production is inhibited by sodium azide, 2,4-dinitrophenol, chloramphenicol, streptomycine etc.[22]

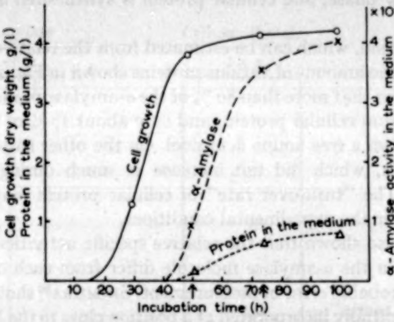


Fig. 1. The relationship between cell growth and α-amylase production in the culture medium. Cell growth was represented as an amount of acetone dry cells isolated from 1 l of the culture medium (about 1,000 cm² medium surface). The amount of protein in the medium was measured as follows: The protein was precipitated from the medium by addition of trichloracetic acid (final 10%), washed twice with 10% trichloracetic acid, washed with ethanol three times and dried in vacuum. ↑: [^{14}C]leucine was added to the medium. ⇞: α-Amylase and bacterial cells were isolated from the medium.

TABLE I

RELATIVE SPECIFIC ACTIVITIES OF [^{14}C]LEUCINE IN THE CULTURE MEDIUM, CELLULAR PROTEIN AND VARIOUS PORTION OF α-AMYLASE

Leucine isolated from:	*Relative specific activity counts/min/μmole*
Culture medium just after an addition of [^{14}C]leucine	38,500*
Culture medium after an incubation for 24 h	36,700*
Cellular protein after an incubation for 24 h	14,000
α-Amylase after an incubation for 24 h	
Whole protein	4,600
Near to the C-terminal residue	7,100
Near to the N-terminal residue	2,700
Band 1**	3,900
Band 2	5,800
Band 3	5,700
Band 4	3,600
Band 5	5,400
Band 6	3,400
Band 7	5,000
Band 8	7,300

Radioactivity sown in this table is corrected by subtraction of a background.

* The contribution from D-[^{14}C]leucine is corrected as follows: True relative specific activity (shown in the table) = observed relative specific activity × 0.5.

** These bands are located from 0.38 l to 0.6 l of the effluent solution of column chromatography. These eight bands, which contain a peptide or peptides containing leucine, are sharp and distinctive as shown in the previous paper[11].

reached the stationary phase, and cellular protein is synthesized *de novo* from a free amino acid pool.

The general situation, which can be estimated from the relative specific activities shown in Table I and the amount of various proteins shown in Fig. 1, is summarized in Scheme 1. It will be seen that more than 80 % of the α-amylase produced after addition of [^{14}C]leucine came from cellular protein, and only about 17 % of the α-amylase was synthesized *de novo* from a free amino acid pool. On the other hand, more than 30 % of the cellular protein, which did not increase so much during this period, was synthesized *de novo*. The "turn-over rate" of cellular protein may be estimated as 15–20 % per 24 h under the experimental conditions.

In Table I, it is also shown that the relative specific activities of [^{14}C]leucine in the various positions in the α-amylase molecule differ from each other to an extent which is beyond the probable error of measurement. SHIMURA[20] showed that a labelled amino acid was preferentially incorporated at a position close to the N-terminal residue of silk fibroin[20]. In the present experiment, it was observed that [^{14}C]leucine was preferentially incorporated close to the C-terminal residue rather than close to the N-terminal residue. But some leucine residues, which may occupy inner positions in the protein molecule, have almost the same relative specific activity as that of the leucine residue close to the C-terminal residue. These results strongly suggest a "stepwise" or "sequential" synthesis of protein from a free amino acid pool.

DISCUSSION

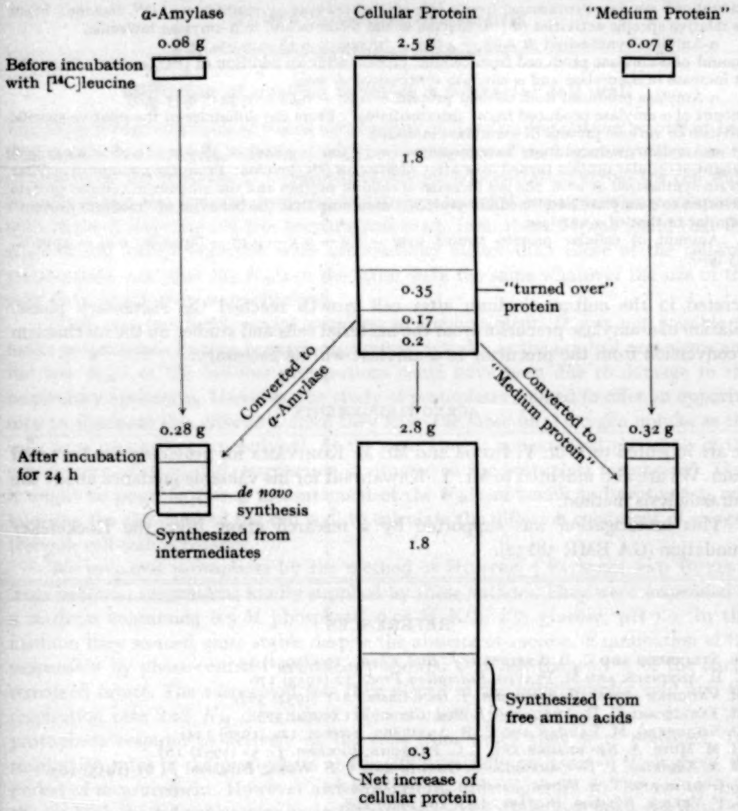
Observations of "unequal" labelling along a peptide chain, which suggest a "stepwise" condensation mechanism, have been presented by some investigators[1–3, 20]. On the other hand, observations which are more readily interpretable in term of a "simultaneous" hypothesis, have also been reported[4–9, 21, 22]. All of these works were performed using animals or animal tissues as an experimental system. There are many interpretations of these results, partially owing to the fact that the experimental system was so complex that it was difficult to evaluate many factors, such as rate of equilibration of intermediates with a free amino acid pool, rate of protein synthesis, turnover rate of protein, etc. It may be considered that some microorganism, especially an amino-acid requiring mutant, would be more suitable for such work.

In these experiments, using the leucine-requiring mutant of *Bacillus subtilis* and α-amylase system, "nonuniform" labelling along a peptide chain was demonstrated. This result is compatible with a "simultaneous" condensation mechanism only when a given amino acid in the protein investigated is replaced at different rates in various positions.

From the relative specific activities recorded in Table I, it was shown that after addition of [^{14}C]leucine about 19 % of the leucine residues close to the C-terminal residue and in fraction 8, and about 7 % of the leucine residue close to the N-terminal residue came from a free amino acid pool. The difference between these values must be attributed to the presence of an "intermediate" form of amino acid during the process of protein synthesis.

Concerning the presence of α-amylase precursor in bacterial cells, some evidence has been presented[19, 23], but it is not yet conclusive. In this experiment, it was concluded that some portion of cellular protein was converted to α-amylase and

SCHEME I


α-Amylase
Cellular Protein
"Medium Protein"
0.08 g
2.5 g
0.07 g
Before incubation with [¹⁴C]leucine
1.8
0.35
"turned over" protein
0.17
0.2
Converted to α-Amylase
Converted to "Medium protein"
0.28 g
2.8 g
0.32 g
After incubation for 24 h
de novo synthesis
Synthesized from intermediates
1.8
Synthesized from free amino acids
0.3
Net increase of cellular protein

The values in this scheme were calculated from the amount of various proteins shown in Fig. 1 and the relative specific activities shown in Table I as follows:

Amount of cellular protein: From the dry weight of the cells and nitrogen content of the cells (10.2 % from Kjeldahl method), together with an assumed value of nitrogen content of proteins (15.5 %).

Cellular protein before incubation = 3.8 × 0.102 ÷ 0.155 = 2.5 (g/l)
Cellular protein after incubation = 4.2 × 0.102 ÷ 0.155 = 2.8 (g/l)

Amount of α-amylase in the culture medium: From the α-amylase activity in the culture medium and enzyme activity of crystalline α-amylase (80,000 $D^{30'}_{40°}$/mg protein N),

α-Amylase before incubation = 1,000 ÷ 80,000 ÷ 0.155 = 0.08 (g/l)
α-Amylase after incubation = 3,500 ÷ 80,000 ÷ 0.155 = 0.28 (g/l)

Amount of protein in the culture medium other than α-amylase ("medium protein"):

From the amount of protein in the culture medium and the amount of α-amylase,
"Medium protein" before incubation = 0.15 — 0.08 = 0.07 (g/l)
"Medium protein" after incubation = 0.60 — 0.28 = 0.32 (g/l)

Amount of cellular protein synthesized from a free amino acid pool after an addition of [^{14}C]leucine:

From the relative specific activities of [^{14}C]leucine in the medium and in cellular protein,

Cellular protein synthesized *de novo* $= 14{,}000 \div 38{,}000 \times 2.8 = 1$ (g/l)

Amount of α-amylase synthesized from a free amino acid pool after addition of [^{14}C]leucine: From the relative specific activities of [^{14}C]leucine in the medium and in α-amylase molecule,

α-Amylase synthesized *de novo* $= 4{,}600 \div 38{,}000 \times 0.28 = 0.034$ (g/l)

Amount of α-amylase produced from cellular protein after an addition of [^{14}C]leucine: From the net increase of α-amylase and α-amylase synthesized *de novo*,

α-Amylase produced from cellular protein $= 0.28 - 0.08 - 0.34 \doteqdot 0.17$ (g/l)

Amount of α-amylase produced from "intermediates": From the difference of the relative specific activities in various protein of α-amylase molecule,

α-Amylase produced from "intermediates" $= (7{,}300 - 2{,}800) \div 38{,}000 \times 0.28 = 0.033$ (g/l)

Amount of cellular protein turned over after addition of [^{14}C]leucine: From the amount of cellular protein synthesized *de novo*, the net increase of cellular protein and the amount of cellular protein converted to α-amylase and "medium protein", assuming that the behavior of "medium protein" is similar to that of α-amylase,

Amount of cellular protein turned over $= 1.0 - 0.3 - 0.17 -$ (approx. 0.2) = approx. 0.35 (g/l)

excreted in the culture medium after cell growth reached the stationary phase. Isolation of α-amylase precursor from the bacterial cells and studies on the mechanism of conversion from the precursor to α-amylase will be necessary.

ACKNOWLEDGEMENTS

We are indebted to Prof. Y. IKEDA and Mr. M. KOHIYAMA for providing the bacterial strain. We are also indebted to Mr. Y. KAWANISHI for his valuable guidance about the hydrazinolysis method.

This investigation was supported by a research grant from the Rockefeller Foundation (GA BMR 58132).

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Short Communications

Diffusion of oxygen through a bacterial cell wall

The oxygen concentration at which suspensions of *Bacillus megaterium* respire at half their maximum rate (K_m) increases with increasing size of the organism[1]. This can be explained by the presence of a gradient of oxygen concentration across the cell wall. In order to test this hypothesis the K_m's of suspensions of *B. megaterium* were compared with those of respiring cell-free preparations made from them. It was found that the K_m's of the intact organism were substantially higher than those of the cell-free preparations and that the K_m's of the latter were the same whatever the size of the cells from which they were obtained.

There was one fundamental weakness in the argument. It was not possible to make preparations having as much respiratory activity as the original organisms and the low K_m's of the cell-free preparations could have been due to damage to the respiratory apparatus. However, the study of protoplasts seemed to offer an opportunity to eliminate this difficulty, since they have the same total oxygen uptake as the cells from which they are derived[2]. At the same time it appears that the whole of the cytochrome oxidase of *B. megaterium* is situated in the protoplast membrane[3]. Thus it might be possible from a measurement of the K_m's of bacilli and protoplasts and knowing the thickness of the cell wall to calculate the diffusion coefficient of oxygen through cell-wall substance.

We prepared protoplasts by the method of HUNTER, CRATHORN AND BUTLER[4] from bacterial suspensions kindly supplied by these authors. They were suspended in a medium containing 0.5 M phosphate, 0.05 M KCl, 1 % glucose, pH 7.0. In this medium they seemed quite stable despite the absence of sucrose. Examination of the suspension by phase-contrast microscopy showed that less than 0.1 % of the bacilli remained intact. The suspension was then placed in the polarographic cell[5] and the respiration rate and K_m determined. Measurements made at 30° showed that the protoplasts respired as actively as the cells from which they were prepared. The respiration rates of the protoplasts and bacilli were fully maintained throughout the period of measurement. However at temperatures differing from 30° by 10° or more the protoplasts did not respire as actively as the parent cells and since the object of the experiments was to overcome the objection that a lowering of K_m could come about by a lowering of respiration rate, only those results obtained with protoplasts as active as the organisms from which they were obtained can be considered. This prevented any measurement of the heat of activation which might have been used to decide whether respiration is diffusion-controlled or not.

We found that the K_m of any protoplast preparation never exceeded that of the suspension of organisms from which it was prepared, but varied between 25 and 100 % of that of the intact parent bacilli. Variations in the K_m of *B. megaterium* could be due to variations in the thickness of the cell wall. It seems unlikely that the affinity of the oxidase involved would vary so as to account for the variable K_m of the protoplasts. The most likely explanation is that the degree of digestion of the cell wall varies in different preparations. We attempted to digest more material from the cells by varying the sodium content and by the addition of trypsin, simultaneously or subsequently, but this did not alter the appearance of the protoplasts nor did it affect the K_m appreciably.

It seems from these results that there is a gradient of oxygen concentration across part of the cell wall of *B. megaterium*, but that this region is removed in a variable amount by digestion.

We are indebted to Drs. Butler, Hunter and Crathorn of the Chester Beatty Research Institute for the supply of bacterial suspensions for advice on the preparation of protoplasts.

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Received May 25th, 1959

The chromatographic purification of pepsinogen

It has been noted in this laboratory that pepsin is strongly adsorbed to DEAE-cellulose under conditions which permit the quantitative elution of pepsinogen. This observation has led to the development of a simple chromatographic procedure for the direct isolation of highly purified pepsinogen from a commercial source of the crude zymogen.

DEAE-cellulose was prepared according to the directions of Peterson and Sober[1] with a final equilibration against 0.1 *M* phosphate buffer, pH 6.8. Commercial (Pentex) swine pepsinogen (1 g) was dissolved in 20 ml of this buffer and applied to a 1.5 cm × 32 cm column of the adsorbent. Gradient elution was accomplished by running a solution of 0.5 *M* phosphate–1.0 *M* NaCl, pH 6.2, through a closed mixing chamber containing 750 ml 0.1 *M* phosphate buffer, pH 6.8. 10-ml fractions were collected automatically at a flow rate of 50 ml/h. A temperature of 4°–7° was maintained during the entire run. All tubes were examined for absorbance at 280 mμ and assayed for actual or potential pepsin activity by the procedure described below.

Pepsin activity and potential pepsin activity were differentiated by taking advantage of the fact that pepsin is readily denatured in alkaline solution (pH > 7) and pepsinogen is rapidly converted to pepsin at pH 2[2]. (a) The potential activity of pepsinogen was determined by first treating 1 ml of the enzyme solution with 3 ml 0.4 *M* borate buffer, pH 8. After an interval of 10 min to insure the denaturation of any pepsin that might be present, the pH was lowered to 2 by adding 1 ml 0.5 *N* HCl. At the end of 10 min, 1 ml of this solution was assayed for peptic activity on hemoglobin[3], and the activity expressed as pepsin units, $[\text{P.U.}]^{\text{Hb}}_{\text{ml}}$, as defined by Anson[3]. (b) The combined activity due to pepsin and pepsinogen was determined

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Abbreviation: DEAE, diethylaminoethyl.

by adding 4 ml of a mixture of 3 parts of 0.4 *M* borate buffer, pH 8, and 1 part of 0.5 *N* HCl to 1 ml of the enzyme solution (final pH of this mixture was 2). After a period of 10 min, 1 ml of this solution was assayed on hemoglobin. Pepsin activity was calculated as the difference between (a) and (b). N analyses, for the purpose of calculating specific activity, $[\text{P.U.}]^{\text{Hb}}_{\text{mg N}}$, were performed by the method of LANNI *et al.*[4].

Fig. 1 shows the chromatographic pattern which was obtained with crude pepsinogen having a specific activity of 0.08 of which 79 % was due to pepsinogen itself and 21 % to pepsin. Pepsin activity could not be detected in any of the fractions in the effluent. The assay for potential pepsin activity, however, identified the second peak as that of pepsinogen.

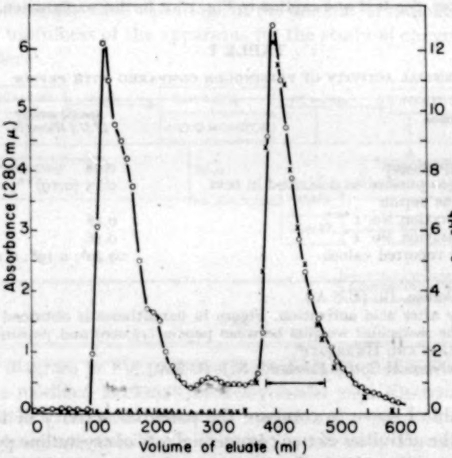

Fig. 1. Chromatographic pattern of crude pepsinogen on a preparative column of DEAE-cellulose. See text for details. Absorbance at 280 mμ indicated by circles, and activity after activation at pH 2 indicated by crosses. Double-headed arrow shows tubes which were pooled for obtaining purified pepsinogen.

The contents of the tubes corresponding to the pepsinogen peak of Fig. 1 were combined and brought to 0.7 saturation with solid $(NH_4)_2SO_4$. The precipitate was collected, dialyzed against distilled water, and finally lyophilized. About 250 mg of dry powder having a potential specific activity of 0.15 was obtained. A 10-mg sample of this preparation was chromatographed on a 0.9 cm × 20 cm column of DEAE-cellulose employing the same technique as before except that the volume of the mixing chamber was reduced to 250 ml and 3-ml fractions were collected at a flow rate of 12 ml/h. As shown in Fig. 2, a single peak was obtained where the points for protein and potential activity fall on the same curve. Chromatography of a sample of the purified pepsinogen which had been subjected to activation at pH 2 yielded no measurable protein which could be eluted from the column under the same conditions. The pepsin so formed could be removed (in a denatured condition) only by elution with 1 % NaOH.

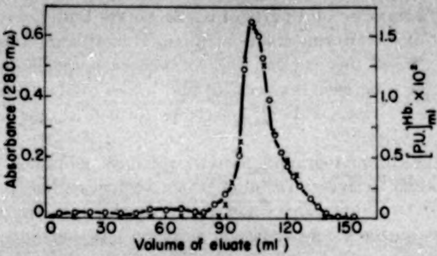

Fig. 2. Chromatographic pattern of purified pepsinogen on an analytical column of DEAE-cellulose. See text and caption to Fig. 1 for further explanation.

TABLE I

POTENTIAL ACTIVITY OF PEPSINOGEN COMPARED WITH PEPSIN

Preparation	*Specific activity [P.U.] Hb/mg N*
Crude pepsinogen*	0.08
Pepsinogen purified as described in text	0.15 (0.19)**
Crystalline pepsin	
Preparation No. 1***	0.18
Preparation No. 2*	0.16
Other reported values	0.20[5]; 0.19[6]; 0.17[7]

* Pentex Inc., Kankakee, Ill. (U.S.A.).

** Based on activity after acid activation. Figure in parentheses is obtained after correcting for the difference in the molecular weights between pepsin (42,000) and pepsinogen (34,500) as reported by VAN VUNAKIS AND HERRIOTT[8].

*** Worthington Biochemical Corp., Freehold, N.J. (U.S.A.).

The data in Table I serve to compare the potential activity of the pepsinogen prepared here with the activities of two commercial lots of crystalline pepsin and with representative values taken from the literature. It is apparent that the simple chromatographic procedure reported here yields a preparation of pepsinogen with a potential activity comparable to that of crystalline pepsin.

Paper No. 4157, Scientific Journal Series, Minnesota Agricultural Experiment Station, University of Minnesota, St. Paul, Minn. This work has been supported by grants from the National Institutes of Health and the National Science Foundation.

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Received June 1st, 1959

Automatic direct quantitation of radioactivity on paper chromatograms

A technique of great usefulness to biochemists involves the use of radioactive compounds as substrates or precursors with the subsequent separation of the radioactive products by paper chromatography. However, the quantitation of the radioactivity in the individual products on the paper chromatogram has been cumbersome and indirect by previously described procedures.

The present report describes an assembly of commercially available components and the modifications in circuitry that are necessary to provide for the automatic direct numerical recording of the amount of radioactivity in spots on paper chromatograms having extremely wide variations in the amount of radioactivity per spot. An example of the usefulness of the apparatus for the study of enzyme reactions will be reported elsewhere[1].

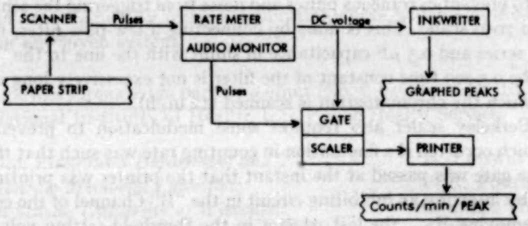

Fig. 1.

The block diagram in Fig. 1 shows the combination of a Nuclear-Chicago Actigraph II with a modified Beckman-Berkeley model 7050 Electronic Counter (scaler) and an unmodified Beckman-Berkeley model 1452 print-out Digital Recorder (printer). The Actigraph consists of a model C-100A Strip Feeder in which the D47 gas flow counting tube (scanner) is equipped with a "micromil" thin window, a model 1620-A Analytical Count-Rate Meter and a Texas Instruments Company Rectiriter chart recorder (inkwriter). The pulses from the scanner go to the rate meter which develops a d.c. voltage proportional to the rate of the radioactivity immediately under the Geiger tube. This voltage is indicated on a front panel meter and is also plotted as a function of time by the inkwriter. In addition the d.c. voltage is used to control an electronic gate in the scaler. The actual pulses produced by the Geiger tube are also fed into the scaler via the rate meter but the counting circuitry is disabled by the gate until the rate of activity exceeds a threshold which in practice is usually set at a gate voltage equivalent to about twice the normal background. Thus when a radioactive spot is scanned, the gate opens when the counting rate is twice the background and the scaler registers the number of counts that accumulate throughout the peak until the rate returns to twice background. At this point the printer scans the scaler decades, prints the total counts in the peak simultaneously drawn by the inkwriter, and then resets the scaler so that it will be ready to integrate the counts when the rate again rises above the threshold. This is all done automatically and it may be

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Abbreviation: d.c., direct current.

emphasized that the counts recorded by the printer will be proportional to the radioactivity in the spot even though the scale of the inkwriter has been greatly exceeded. In practice, the range of the rate meter is set at 300 counts/min and the time constant is set at 50 sec. Thus most of the peaks are offscale on the inkwriter, which serves only to locate the spots and facilitate their correlation with counts recorded by the printer. This correlation is also expedited by placing standard spots of radioactive ink as reference points at the beginning of each section of the paper strip.

The following modifications are required in the above mentioned equipment.

1. The pulses for the scaler are taken from pin 3 of V_8, the audiomonitor driver of the rate meter, through a cable to the Channel A connection of the scaler. These pulses are 30 V high at this point and more than adequate to drive the counter.

2. The d.c. gate-control voltage for the scaler is obtained from pin 3 of V_{5A}, the cathode follower feeding the rate-indicating volt meter in the 1620A rate meter and is carried through a cable attached to Channel B of the scaler. It is necessary to filter this voltage to prevent extraneous pulses and noise from triggering the sensitive gating circuit in the 7050 scaler. This is done by connecting a low-pass filter, one megohm resistance in series and 0.5 μF capacitance in shunt with the line to the "B" input of the scaler. The 0.5-sec time constant of the filter is not excessively long compared to the rate at which the chromatogram is scanned (12 in./h).

3. The Berkeley scaler also requires some modification to prevent a loss of triggering which occurred if a fluctuation in counting rate was such that the threshold voltage of the gate was passed at the instant that the printer was printing. This can be corrected by inserting an inhibiting circuit in the "B" Channel of the counter. This is done by removing R_{319}, the last resistor in the threshold-setting voltage divider, from the — 60 V line and connecting it to pin 6 of V_{205}, the reset generator. This point in the circuit rises to + 60 V when a count is completed and stays at this level while the printing operation is performed. This is sufficient voltage to inhibit the "B" Channel during this time. In order to make the resulting voltages compatible with the circuitry in the B Channel it is necessary to change R_{319} from 120 kΩ to 97 kΩ. The center tap of R_{318}, the threshold-setting potentiometer, is removed from ground, and capacitor C_{310} is changed from 0.01 to 0.5 μF to insure against spurious triggering of the B Channel by pulses occurring in the control circuitry during the printing operation. The printer requires no modification.

Thus with the above minor modifications of commercially available equipment an instrument is obtained which scans a paper strip for radioactivity, records the rate on a moving chart, and records the total number of counts in each peak of radioactivity on the paper regardless of the counting rate. A number of strips can be taped together up to a length of 50 ft. and the instrument will function without attention for a period of 2 days.

For chromatograms with poorly resolved peaks it is possible to print out the counts in fixed intervals of time by connecting an Industrial Timer Corporation type CM-4 recycling timer to close a microswitch and apply a 1.5 V battery to the input of the "B" Channel for the specified time at the end of which the voltage is momentarily removed to close the gate and print the count accumulated in that period.

The calibration of the equipment in terms of counting efficiency and precision is an operation that will need to be carried out for each installation and each combination of paper, solvent system and threshold setting. In this laboratory Whatman No. 1

filter paper and completely volatile solvents have been used. A slit-width of 0.25 in and a scanning rate of 12 in/h were used throughout. Under these conditions the printer prints out counts/1.25 min/spot. The system as described includes background only after the gate has opened and does not include the radioactivity in that part of the peak below the threshold. These two factors require corrections of opposite sign and there is a tendency for them to cancel each other, with a net loss of about 100 counts/min per peak when the threshold is set at twice the background count. In addition to this correction, there is a percentage loss that results from the geometry, the window, and the absorption losses caused by the filter paper. These 3 factors combined to give an efficiency that varied from 37 to 45 % when the printed counts were compared with counts/min from a windowless gas-flow counter calculated for samples at infinite thinness over a range of 500 to 20,000 counts/min. These data were independent of the time of development in solvent over a range of 3 to 9 h. It is clear that the significance of the 100 counts/min correction increases when peaks contain less than 1000 counts/min and decreases at higher levels and that maximum accuracy can be obtained by using a calibration curve which empirically combines all factors for any given system.

This work was supported in part by a grant (No. C-646) from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

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The McArdle Memorial Laboratory
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Received June 1st, 1959

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The isolation of iminazole acetaldehyde as the only metabolite of the action *in vitro* of placental histaminase on histamine

In a recent paper[1] a chemical synthesis of the 2,4-dinitrophenylhydrazone of iminazole acetaldehyde was reported as well as the identification of iminazole acetaldehyde as the only metabolite of the action *in vitro* of histaminase from hog kidney on histamine. Since the literature concerning the enzymic destruction of histamine by oxidative deamination has recently become very involved[2–9], it appeared to be important to investigate whether human placental histaminase would yield during its action on histamine *in vitro* the same metabolite as hog kidney.

For the preparation of a placental-histaminase extract a normal placenta, soon after birth, was mechanically minced to a fine brei, and to the weighed wet pulp was added with mechanical stirring the double amount of 1 % NaCl. After straining through muslin the pulp was extracted for a second time with the same amount of 1 % NaCl as before. The combined filtrates were centrifuged, and to the clear, measured supernatant 42.4 g $(NH_4)_2SO_4$/100 ml (0.6 satn.) was added with mechanical stirring. To the precipitate formed, filtered over fluted filters, 200 ml 0.02 *M* phosphate buffer, pH 6.8, were added. The slightly turbid solution was dialysed against tap water for 2.5 h. This highly active enzymic extract was used for the study of the action of placental histaminase on histamine. By means of various techniques, applied in investigations of histaminase from hog kidneys[1], it was found in the present work that by the action on histamine of placental histaminase like of that of hog kidney histaminase only one metabolite, an iminazole derivative, is formed. In paper-chromatographic assays the metabolite gave a single diazo-stained spot, which clearly showed characteristics suggestive of iminazole acetaldehyde. When subjected to oxidation, either enzymic with pure xanthine oxidase (60 min, 37°) or non-enzymic with 0.1 *N* $KMnO_4$ (5 min, 50°), the product yielded iminazole acetic acid. As in previous experiments, an attempt was made to identify this reaction product of the action of placental histaminase on histamine by the isolation of one of its derivatives.

Reaction mixtures in 100-ml Erlenmeyer flasks each contained 10 ml of the dialysed placental extract, described above, 4 ml phosphate buffer (pH 6.8), 0.8 ml of a diluted solution of catalase (L. Light, Ltd.), corresponding to about 0.6 mg catalase, and 4.4 ml of a histamine solution containing 44 mg histamine dihydrochloride. Three such reaction mixtures containing in total 132 mg histamine dihydrochloride or 79.6 mg histamine base, were separately incubated at 37° for 75 min. The combined incubation mixtures were brought into reaction with 2,4-dinitrophenylhydrazine according to the method previously described. The orange-red crystals which formed were filtered and washed with 50 % alcohol (m.p. of dried crystals was 112°). On recrystallisation from 50 % acetone, 189.2 mg of golden silky needles (m.p. 124–126°) were obtained, very similar to iminazole acetaldehyde 2,4-dinitrophenylhydrazone, prepared in previous work[1] by two different routes, a synthetic and an enzymic one. Since the m.p. of the new preparation did not change on further recrystallisation from 50 % alcohol or 50 % acetone and remained undepressed by the admixture of preparations of iminazole acetaldehyde 2,4-dinitrophenylhydrazone of either synthetic or enzymic origin, or by the simultaneous admixtures of both, the further purification on bentonite-kieselguhr (4:1) columns, used previously, appeared to be superfluous. For a further identification of the compound a paper chromatogram was prepared in parallel with that of the authentic preparation of synthetic iminazole acetaldehyde 2,4-dinitrophenylhydrazone and also with one of enzymic origin. Fig. 1 shows the completely identical paper-chromatographic behaviour of the three compounds and of a mixture of these substances. In all instances yellow spots were obtained, R_F 0.86 (propanol–ammonia, Whatman No. 4 paper, developing time 5 h). Identical results with all these preparations were also obtained in another solvent system (R_F 0.62(10 % Glycerol v/v, 10 % acetic acid v/v, 10 % phenol w/v, Whatman No. 1 paper). Finally, since 189.2 mg of the pure 2,4-dinitrophenylhydrazone of iminazole acetaldehyde have been isolated from 79.6 mg histamine (91 % of the theoretical yield) this seems to indicate that the placental histaminase has changed histamine with the stoichiometric formation of a single metabolite, iminazole acetaldehyde.

Recently, LINDAHL, LINDELL, NILSSON, SCHAYER AND WESTLING[10] studied the metabolism of $[^{14}C]$histamine in plasma during human gestation. On the basis of results obtained by radioisotope methods, applied to plasma of women during their last weeks of pregnancy, these workers suggest that human pregnancy plasma inactivates histamine enzymically by oxidative deamination, and that methylation of histamine which SCHAYER AND COOPER[11] supposed to be the principal route of histamine metabolism in man is apparently not the route of histamine degradation by pregnancy plasma.

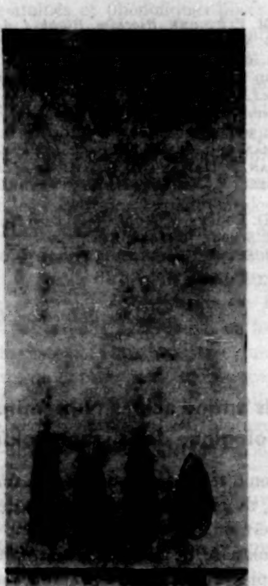

Fig. 1. Paper chromatogram of the iminazole acetaldehyde 2,4-dinitrophenylhydrazone. S, synthetic compound; P, compound obtained by enzymic action of placental extracts; K, compound obtained by enzymic action of hog-kidney extract; Mix., mixture of S, P, and K.

The evidence reported in the present paper of the stoicheiometric formation of a single metabolite, identified as iminazole acetaldehyde, in the attack *in vitro* of histaminase from placenta (the site of histaminase formation in human pregnancy[12,13]) on histamine seems to indicate that in human pregnancy histamine is being destroyed mainly by oxidative deamination.

My sincere thanks are due to Dr. C. P. STEWART for his continued interest in this work and to Prof. R. KELLAR, Simpson Memorial Maternity Pavilion, Royal Infirmary, Edinburgh, for kind permission to obtain the clinical material. I am most grateful to Dr. R. C. BRAY, Chester Beatty Research Institute, London, for a generous gift of pure xanthine oxidase and to Dr. H. TABOR, Bethesda, Maryland, for a kind supply

of crystalline iminazole acetic acid. I should also like to thank Dr. M. Fletcher for her assistance early in this work.

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Received May 15th, 1959

Biochim. Biophys. Acta, 37 (1960) 527–530

Signification des amino acides N-terminaux pour l'action biologique de la staphylokinase

Dans une note antérieure on a relaté les résultats de l'analyse chimique d'un préparé de staphylokinase purifiée, de même que les résultats des déterminations des amino acides N-terminaux et C-terminaux[1].

On sait que la staphylokinase, de même que la streptokinase, sont des activateurs du plasminogène, protéine plasmatique, inactive et qui, sous l'action de ces activateurs, est convertie en plasmine, enzyme à action fibrinolytique.

L'action activante a été interprétée, soit comme le résultat d'une combinaison stoechiométrique directe entre l'activateur et le plasminogène[2], soit comme une réaction enzymatique en deux étapes[3], la première étape comportant une réaction entre l'activateur d'origine bactérienne (staphylokinase, streptokinase) et un cofacteur plasmatique. Cette réaction réalise la formation d'un autre activateur, qui, dans une deuxième étape, transforme par voie enzymatique le plasminogène en plasmine.

Le mécanisme d'activation par la staphylokinase comporte en tout cas une réaction intermédiaire entre ce facteur enzymatique et son substratum.

Tenant compte de la structure protéique de la staphylokinase et du fait que les amino acides terminaux, par leur position spéciale dans la chaîne polypeptidique, pourraient intervenir de manière directe dans le comportement chimique et biologique de ce facteur enzymatique, nous avons entrepris l'étude de l'influence du blocage des aminoacides N-terminaux sur l'action activante spécifique exercée par la staphylokinase sur le plasminogène. La présente note expose les résultats de cette étude.

Les amino acides N-terminaux, identifiés dans la staphylokinase purifiée, ont

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Abbréviation: DNFB, dinitrofluorobenzène; DNP, dinitrophényl.

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été: la glycine, la leucine, l'alanine[1]. Rapporté à un poids moléculaire minimum égale à 35,000, le calcul nous indique l'apparition dans la molécule de la staphylokinase d'un seul résidu N-terminal pour chacun de ces amino acides.

L'étude de l'influence des amino acides N-terminaux sur l'action activante de la staphylokinase a été poursuivie en bloquant par acétylation successive et progressive les groupements α-aminés, ε-aminés et phénoliques libres. Ce blocage sélectif a pu être réalisé en utilisant des quantités très petites et rigoureusement contrôlées d'anhydride acétique[4]. La manipulation des petites quantités d'anhydride acétique, qui sont réclamées pour un blocage sélectif des groupements aminés libres, a été facilitée en utilisant le benzène comme milieu de dissolution. On travaille en présence de l'acétate de sodium (à demi-saturation à 0°) selon la technique classique d'acétylation[5].

L'acétylation est contrôlée pour les groupements aminés à l'aide de la réaction à la ninhydrine et pour les groupements phénoliques à l'aide du réactif Folin à pH 8.

Dans le but de préciser les conditions expérimentales réalisant le seul blocage des groupements NH_2 des amino acides N-terminaux, il était nécessaire de disposer d'un moyen de contrôle sélectif de l'évolution du processus de ce blocage. Ce moyen de contrôle a été réalisé par l'intermède de la réaction au dinitrofluorobenzène. À l'aide de cette méthode, on a pu confirmer l'observation de REID[4] que le blocage par acétylation a lieu de manière sélective et progressive, en fonction de la concentration en anhydride acétique dans le système. REID[4] constate que les groupements α-aminés des amino acides N-terminaux de l'hormone de croissance sont les premiers à réagir avec l'anhydride acétique, de sorte qu'en utilisant de faibles quantités de cet agent acétylant, on réalise de manière sélective le seul blocage de ces groupements. Le blocage des groupements ε-aminés a lieu seulement pour des concentrations plus fortes en anhydride acétique.

Dans le cas de la staphylokinase, nous avons trouvé comme quantité limite, acétylant les seuls groupements aminés des amino acides N-terminaux, la quantité de 2 μl/100 mg de staphylokinase. L'augmentation progressive de la quantité d'anhydride acétique réalise le blocage successif des autres groupements NH_2 libres appartenant aux amino acides intracathénaires ainsi qu'aux groupements phénoliques libres (voir Tableau I).

Le blocage successif est indiqué par la disparition successive de leur capacité réactionnelle (réaction à la ninhydrine pour les groupements NH_2, réactif Folin pour les groupements phénoliques). Chaque étape de l'acétylation de la staphylokinase, donc du blocage de ses groupemnets NH_2 et phénoliques libres, est contrôlée du point de vue de la capacité de la staphylokinase ainsi modifiée, de réaliser la transformation du plasminogène en plasmine.

Le protocole d'une expérience peut être résumé comme suit: 100 mg de staphylokinase sont traités avec des quantités d'anhydride acétique variant entre 2 et 20 μl. De chaque système, on prélève des échantillons, sur lesquels on pratique les contrôles suivants: (a) Présence et dosage des groupements NH_2 libres (à l'aide de la ninhydrine). (b) Présence et dosage des groupements phénoliques libres (à l'aide du réactif Folin à pH 8). (c) Traitement par DNFB pour le contrôle de l'évolution du blocage par acétylation des groupements α-NH_2. La séparation des DNP-amino acides terminaux et des DNP-amino acides intracathénaires s'effectue par extraction dans de l'éther, des produits de l'hydrolyse acide, les premiers étant solubles dans ce dissolvant. L'identification et le dosage des DNP-amino acides terminaux s'effectue sur l'extrait éthéré par chromatographie sur papier dans le système phtalate–alcool amylique tertiaire[6]. La totalité des DNP-amino acides intracathénaires est déterminée après extraction dans le *n*-butanol et dosage photométrique de ces extraits. L'activité de chaque échantillon est enfin mesurée selon la technique classique[7] par comparaison avec l'échantillon de staphylokinase non soumise à acétylation. Le Tableau I résume nos expériences.

TABLEAU I

CORRÉLATION ENTRE L'ACTION BIOLOGIQUE DE LA STAPHYLOKINASE ET LE BLOCAGE PAR ACÉTYLATION DES AMINO ACIDES N-TERMINAUX

Echantillon No.	*µl anhydride acétique/100 mg staphylokinase*	*Activation du plasminogène par la staphylokinase*	*Groupements NH_2 libres (exprimés en mequiv. leucine/100 mg staphylokinase*	*Groupements phénoliques libres (exprimés en mequiv. tyrosine/100 mg staphylokinase)*	*Groupements N-terminaux libres*	*Extraits butanoliques des DNP-amino acides intracathénaires (exprimés en transmissions)*
1	0	+++	$2.2 \cdot 10^{-2}$	$1.35 \cdot 10^{-2}$	Présents	32
2	2	0	$1.4 \cdot 10^{-2}$	$1.30 \cdot 10^{-2}$	Absents	33
3	8	0	$0.6 \cdot 10^{-2}$	$0.56 \cdot 10^{-2}$	Absents	65
4	20	0	0	$0.06 \cdot 10^{-2}$	Absents	70

L'échantillon No. 1 est constitué par le système témoin contenant la staphylokinase en absence de l'anhydride acétique, mais contrôlé dans des conditions absolument identiques au système additionné d'anhydride acétique.

L'échantillon No. 2 est constitué par le système contenant 2 µl d'anhydride acétique, quantité limite acétylant de manière sélective les amino acides N-terminaux, donc bloquant leurs groupements NH_2 libres. Dans ce cas, la valeur de l'extinction de l'extrait butanolique des DNP-amino acides intracathénaires est très proche de la valeur correspondant à l'échantillon de staphylokinase .Dans ces conditions, on constate la suppression totale de l'activité biologique de la staphylokinase, ce qui indique la nécessité des groupements NH_2-terminaux libres pour son activité.

En augmentant la quantité d'anhydride acétique, on réalise aussi le blocage des groupements ε-NH_2 et des groupements phénoliques libres.

L'acétylation sélective des groupements N-terminaux confirme l'hypothèse du rôle spécial de ces groupements dans la molécule, rôle qui se manifeste tant dans le comportement chimique que dans le comportement biologique de la staphylokinase. Par l'acétylation des groupements N-terminaux, la staphylokinase n'exerce plus son action activante sur le plasminogène et sa transformation en plasmine, enzyme à action fibrinolytique, n'a plus lieu.

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Reçu le 14 Avril, 1959

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The synthesis of amino acids by the perfused cow udder

From experiments with isotopic tracers, carried out on living goats, it was concluded that free glutamic acid, glutamine and asparagine are taken from the blood stream by the lactating mammary gland and are used in the biosynthesis of casein[1,2]. These free amino acids appear to be practically the only precursors of the glutamic acid, glutamine and asparagine residues of casein. Conversion of glutamine into glutamic acid and of asparagine into aspartic acid occurred only on a very small scale, although rapid formation of plasma glutamine from glutamic acid was observed. From these experiments *in vivo* it was not possible to conclude whether or not the conversion of glutamic acid into glutamine occurs in the mammary gland.

It has been shown previously that non-essential amino acids are synthesised in the isolated cow udder after administration of [1-^{14}C]butyrate[3] and [1-^{14}C]propionate[4] to the perfusion blood. Milk casein synthesized during perfusion was hydrolysed with acid and the isolated glutamic acid and aspartic acid obtained showed the highest specific activities. However, no information was obtained in these experiments concerning the relative distribution of specific activities between glutamic acid and glutamine, or between aspartic acid and asparagine because of the conversion of asparagine and glutamine into aspartic acid and glutamic acid, respectively, which occurs when proteins are hydrolysed with acid.

In the experiments reported here, milk casein samples synthesised in the [1-^{14}C]-butyrate and [1-^{14}C]propionate perfusion experiments, were submitted to enzymic hydrolysis using a pancreas suspension as described by BARRY[1]. The amino acids were separated from the casein digest by dialysis and the diffusate was freeze-dried. Aspartic acid and glutamic acid were separated on a Dowex 1 column[5]. The first peak eluted from the column contained glutamine and asparagine. These fractions were evaporated to dryness *in vacuo*, the residue was dissolved in 200 ml 1 *N* HCl and hydrolysed for 1 h. The resulting aspartic acid and glutamic acid were separated by elution from a Dowex 1 column as previously described. The amino acids isolated proved to be pure as tested by paper chromatography. The specific activities were measured as previously described[6].

TABLE I

SPECIFIC ACTIVITIES OF AMINO ACIDS ISOLATED FROM MILK CASEIN AFTER PERFUSION OF THE ISOLATED COW UDDER

[1-^{14}C]propionate and [1-^{14}C]butyrate were administered to the perfusion blood and the milk casein hydrolyzed enzymically and by acid. All values are expressed as means ± standard error of the mean.

Amino acid isolated	*[1-^{14}C]propionate counts/min/mg carbon*	*[1-^{14}C]butyrate counts/min/mg carbon*
A. Enzymic hydrolysis		
Aspartic acid	6920 ± 105	2180 ± 33
Asparagine	1970 ± 20	695 ± 10
Glutamic acid	2980 ± 30	4390 ± 45
Glutamine	2300 ± 23	2485 ± 25
B. Acid hydrolysis		
Aspartic acid	3780 ± 57	1490 ± 30
Glutamic acid	2640 ± 26	3425 ± 35

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The results in Table I show that in both perfusion experiments the specific activity of aspartic acid was some 3 times that of asparagine. The specific activity of glutamic acid was 1.3 and 1.8 times that of glutamine in the $[1\text{-}^{14}C]$propionate and $[1\text{-}^{14}C]$butyrate experiments, respectively. From these results it can be concluded that glutamic acid is acting as a precursor of glutamine, and aspartic acid of asparagine in the cow udder, the first reaction proceeding more rapidly than the second. Previous work has shown that glutamine and glutamic acid and asparagine and aspartic acid are not interconverted during or after their incorporation into casein[1,2]. These compounds should be considered as 4 distinct non-essential amino acids. The appreciable differences recorded here in the specific activities of the amino acids of each group after enzymic hydrolysis also show that casein asparagine and aspartic acid, glutamine and glutamic acid have different origins.

In each experiment, the specific activities of glutamic and aspartic acids from the acid casein hydrolysates were in between those of glutamic acid and glutamine, and of aspartic acid and asparagine, respectively.

In previous work in which casein was hydrolysed with acid, aspartic acid showed a greater labelling than glutamic acid in the presence of $[1\text{-}^{14}C]$propionate. Opposite results were obtained after the addition of $[1\text{-}^{14}C]$butyrate or $[1\text{-}^{14}C]$acetate[4]. These observations have been confirmed in the experiments reported here, the differences between the specific activities of aspartic acid and glutamic acid being more striking, however, after enzymic hydrolysis.

This work was supported by a grant from the Belgian I.R.S.I.A. Foundation.

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Received April 3rd, 1959

Biochim. Biophys. Acta, 37 (1960) 533–534

Competitive inhibition of D-amino acid oxidase by benzoate as a function of substrate

For present purposes competitive inhibition may be summarized briefly as follows: The enzyme, E, bears a site at which either substrate, S, or inhibitor, I, combines reversibly to form an enzyme-substrate complex, ES, or an enzyme-inhibitor complex, EI, respectively. The reactions involved are $E + S \rightarrow ES \rightleftharpoons E + P$ (a), where P is the product derived from S by enzyme action, and $E + I \rightleftharpoons EI$ (b). The quantitative relationships between the components involved in (a) are expressed by $(E)(S)/(ES) = K_s$ (c) and $(E)(I)/(EI) = K_i$ (d), where K_s and K_i are constants and the parentheses indicate concentrations. The constants are usually evaluated by means of the equation $1/v = [1/V_m][K_s + K_s(I)/K_i][1/S] + 1/V_m$ (e), where v, which is considered directly

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proportional to (ES), is the rate of formation of P in the presence of rate-limiting (S) and V_m is the maximum rate. Plots of $1/v$ against $1/(S)$ gives straight lines with the same intercept of the $1/v$ axis. The intercept is $1/V_m$. When I is not present the second term within brackets equals zero and the slope of the line measures K_s. In the presence of I the slope is a function of both constants. Conformation of experimental data to (e) is conventionally taken to indicate competitive inhibition.

The foregoing implies that the value of K_i should be independent of the particular substrate used in its estimation. However, in the present work, which has been reported in preliminary form[1], the competitive inhibition of D-amino acid oxidase by benzoate[2,3] was studied as a function of different substrates and, contrary to expectation, the value of K_i was found to depend upon the substrate used in the estimation of the constant.

Aqueous extracts of acetone-dried pig kidney were used as preparations of the oxidase. The D- and DL- form of each amino acid tested was used, except for the proline which was employed as the DL-mixture only. Two samples of each substrate were used; one was obtained from Nutritional Biochemicals Corporation, the other from California Corporation for Biochemical Research. The oxidation of substrate by the enzyme was followed manometrically[4].

In a number of tests four levels of substrate alone and three levels of inhibitor in the presence of three of substrate were employed. Estimations of K_s and K_i were made conventionally from plots of $1/v$ against $1/(S)$. In other tests three levels of substrate alone and three levels of inhibitor plus one of substrate were employed. In these tests two amino acids were studied simultaneously. In addition to the constants, these tests yielded relative values of V_m. Representative tests of the first kind are given in Fig. 1. These tests, by usual interpretation and in agreement with earlier tests with alanine[2,3], indicate that benzoate inhibits the oxidase by competing with substrate.

The means of all values of K_s, K_i, and relative values of V_m are given in Table I. At least three levels of K_i are apparent, one obtained with alanine or proline, another with valine, isoleucine, or phenylalanine, and a third with methionine. The levels of K_i do not appear correlated with the corresponding values of K_s or V_m.

The present study does not provide definitive explanation of the variation of K_i with substrate. However, one possibility bears consideration: The inhibitor may react only with the enzyme–substrate complex with the liberation of substrate, *i.e.*, instead of (b), reaction $ES + I \rightleftharpoons EI + S$ (f) occurs. This reaction in contrast to (b) implies that the formation of EI depends upon the particular ES involved. If (f) obtains, the inhibitor constant is described by $(ES)(I)/(EI)(S) = K_{is}$ (g), rather than (d). The equation relating rate of reaction to (S) is then the same as (e) except that the second term within the brackets, $K_s(I)/K_i$, is replaced by $(I)/K_{is}$. If both (b) and (f) occur, two species of enzyme–inhibitor complex are implied, and the second term is $(I)[K_s/K_i + 1/K_{is}]$. Since each of the terms reduce to $(I)c$, where c is a constant, the same rate equation applies to each possibility. It follows from this that data conforming to conventional requirements for competitive inhibition, but obtained by use of one substrate, can not define the reaction(s) involved in the inhibition. If more than one substrate is used and the values of K_i obtained are all the same, it is reasonable to infer that reaction (b) is involved. If K_i varies with substrate, it seems reasonable to infer that reaction (f) occurs. The present results with the

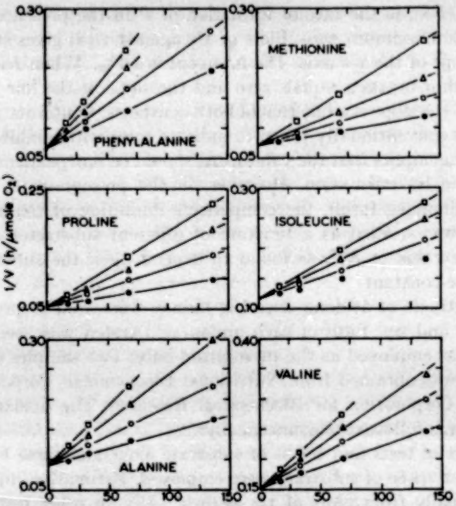

Fig. 1. Inhibition of D-amino acid oxidase by benzoate. The solid circles indicate the results obtained in the absence of inhibitor. The open circles, trangles, and squares indicate respectively the presence of 10^{-4}, $2 \cdot 10^{-4}$, and $4 \cdot 10^{-4}$ *M* benzoate in the case of methionine and $5 \cdot 10^{-5}$, 10^{-4}, and $1.5 \cdot 10^{-4}$ *M* benzoate in the tests with the other amino acids.

TABLE I

COMPETITIVE INHIBITION OF D-AMINO ACID OXIDASE BY BENZOATE AS A FUNCTION OF SUBSTRATE

The values given are means. The values of K_s and K_i bear standard deviations of about 5 %.

Amino acid	*Relative V_m*	K_s *(M)*	K_i *(M)*
Alanine	1.00	$6.3 \cdot 10^{-3}$	$5.9 \cdot 10^{-5}$
Proline	1.66	$5.8 \cdot 10^{-3}$	$7.0 \cdot 10^{-5}$
Phenylalanine	1.39	$14.0 \cdot 10^{-3}$	$9.2 \cdot 10^{-5}$
Valine	0.60	$4.6 \cdot 10^{-3}$	$9.6 \cdot 10^{-5}$
Isoleucine	0.79	$4.1 \cdot 10^{-3}$	$10.4 \cdot 10^{-5}$
Methionine	1.32	$5.3 \cdot 10^{-3}$	$16.3 \cdot 10^{-5}$

oxidase appears to demand reaction (f). However, there is indication that the oxidase combines with competitive inhibitors in the absence of substrate[5]. Therefore, both reaction (b) and (f) may occur.

Some support for the above considerations is provided by the non-competitive reversible inhibition of hexokinase by glucose-6-phosphate[6]. As indicated for benzoate, the value of the inhibitor constant for the phosphate was found to depend upon the substrate used in its determination. Discussion of mechanisms that may account for the finding is presently not in order; however, the finding does suggest reaction between the phosphate and kinase–substrate complex and that the extent of the reaction depends upon the substrate involved.

This work was supported by the U.S. Atomic Energy Commission.

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Received May 1st, 1959

Biochim. Biophys. Acta, 37 (1960) 534–537

The effect of carnosine and related compounds on the respiration of frog-skeletal-muscle homogenates*

SEVERIN *et al.* have reported that carnosine and anserine, when added to crude muscle homogenates, augment the utilization of oxygen, the breakdown of glycogen and the formation of ATP, creatine phosphate and various phosphate esters. These studies have been reviewed elsewhere[1–3]. SHERSTNEV[4] claimed that these findings were due to pH effects. JENCKS AND HYATT[2] reported that carnosine stimulates glycolysis in dialyzed muscle extracts, but only in the absence of EDTA, and suggest that carnosine removes interfering heavy metals from the system by chelation. The present communication is concerned with a further study of the effect of carnosine and related compounds on oxygen consumption by muscle homogenates. The experimental conditions were similar to those described by SEVERIN.

Table I shows that carnosine has no effect on the O_2 uptake of crude frog-muscle homogenates either in the presence or absence of ATP, AMP and EDTA. In addition, no effect was observed after 2-h incubation. No synthesis of creatine phosphate or uptake of inorganic phosphate was observed in these experiments. These findings are in contrast to the results of the Russian workers. The crude homogenates contained about 5 mg/100 ml endogenous carnosine. It is possible that this is sufficient to satisfy the carnosine requirement of the system.

Several compounds structurally similar to carnosine (acetylhistidine, propionylhistidine, anserine, histidine and carnosine methyl ester) were studied as possible carnosine antagonists. Only carnosine methyl ester had a measurable effect. Table II shows that the rate of O_2 uptake remains linear for a much longer period of time in the presence of the ester as compared to the control. Carnosine methyl ester was partially hydrolyzed to carnosine and methanol during the course of the experiment, but methanol was shown to have no effect on the rate of O_2 consumption. It is possible that the ester is (a) more readily converted to a metabolically active form of carnosine

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Abbreviations: ATP, adenosine triphosphate; AMP, adenosine monophosphate; EDTA, ethylenediaminetetraacetic acid.

* Taken in part from a dissertation submitted to the Graduate School of Arts and Sciences of the University of Buffalo in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1958, by C. J. PARKER, JR.

TABLE I

THE EFFECT OF CARNOSINE ON THE RESPIRATION OF FROG-MUSCLE HOMOGENATES

Frog leg muscle was homogenized in a Waring blendor for 30 sec in 3 vol 0.1 *M* tris(hydroxymethyl) aminomethane, pH 7.4, containing KCl (0.12 *M*), NaCl (0.016 *M*), $MgCl_2$ (0.014 *M*) and EDTA (0.002 *M*). The homogenate was comminuted further in a loose-fitting glass homogenizer. Each flask contained 1.0 ml homogenate, 2.0 ml of the above buffer without EDTA, 9 µmoles phosphate, 10 µmoles creatine, 10 µmoles ATP or AMP in the side arm where indicated, and water to give a final vol. of 4.0 ml. The center well contained 0.2 ml 10% NaOH on a filter paper wick. O_2 uptakes are expressed as μlO_2/mg tissue (dry wt.).

	No. of Expts.	*O_2 uptake after 60 min**
Homogenate, ATP, 5 µmoles carnosine	3	4.44
Homogenate, ATP	3	4.74
Homogenate, ATP, 5 µmoles carnosine without EDTA	1	4.24
Homogenate, ATP, without EDTA	1	4.42
Homogenate, AMP, 5 µmoles carnosine	2	2.86
Homogenate, AMP	2	2.68
Homogenate, 5 µmoles carnosine	2	1.02
Homogenate	2	1.16

* Values are reliable to within 10%.

TABLE II

THE EFFECT OF CARNOSINE METHYL ESTER ON THE RESPIRATION OF FROG-MUSCLE HOMOGENATES

Conditions are identical to those described in Table I. The rates of O_2 utilization are expressed as μlO_2/h/mg tissue (dry wt.).

	Rate of O_2 uptake			
	Initial	*100 min*	*120 min*	*160 min*
Homogenate, ATP	3.9	2.0	1.8	1.0
Homogenate, ATP, 5 µmoles ester	3.5	3.5	2.9	2.4
Homogenate, ATP, 5 µmoles methanol	3.9	2.4	1.8	0.8

than is free carnosine in the systems used or (b) is antagonistic to the action of free carnosine, which, in turn, may inhibit O_2 uptake.

It may be concluded from these studies that free carnosine has no measurable effect on oxygen uptake of crude muscle homogenates. It is felt, however, that the action of the ester does suggest a possible new approach to the question of the role of carnosine in muscle.

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Received May 19th, 1959

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Some properties of ornithine δ-transaminase from *Neurospora*

Ornithine δ-transaminase has been demonstrated in crude extracts of *Neurospora*[1] and of various other microorganisms[2], as well as in tissues of animal[3] (*cf.* QUASTEL AND WITTY[4]) and plant[2] origin; the occurrence of this enzymic activity follows a taxonomically distinctive pattern in the organisms tested[2]. The transaminase, which can interconvert ornithine and glutamic γ-semialdehyde (with a concomitant interconversion of α-ketoglutarate and glutamate), has been recognized as an enzyme of arginine biosynthesis in *Neurospora*[5,6]. The present report is concerned with a partial purification and a brief characterization of the transaminase from this organism.

Mycelium of wild-type *N. crassa*, strain 74A[7], was grown on minimal Medium N[8] in stationary culture at 30° for 3 days and was extracted by grinding with cold 0.1 *M* potassium phosphate buffer, pH 7.9, made 2 m*M* in glutathione. The resulting extract was clarified by centrifugation at about 5000 × *g* and treated with $(NH_4)_2SO_4$ at 0°. The fraction obtained between 35 and 70 % was collected, dissolved in buffer, and further fractionated with $(NH_4)_2SO_4$. The precipitate formed between 45 and 55 % was then collected and dissolved in and dialyzed against glutathione-containing buffer. An approximately 6-fold enzyme purification with an over-all yield of 20 % was achieved.

Transaminase activity was measured colorimetrically through the reaction of the glutamic γ-semialdehyde produced with *o*-aminobenzaldehyde[1,9]. Protein was determined by the method of LOWRY *et al.*[10]. Standard conditions for the enzyme tests were as follows: the complete system contained, in a final vol. of 1.5 ml, 0.15 mmole potassium phosphate, pH 7.4, 7.5 μmoles L-ornithine monohydrochloride, 7.5 μmoles α-ketoglutaric acid (neutralized with sodium carbonate), 0.025 μmole pyridoxal phosphate, 1.5 μmoles glutathione (for enzyme stabilization), and enzyme solution (about 0.5–1 mg protein). Incubation was carried out at 37° for 30 min.

The activity of the purified enzyme without added pyridoxal phosphate (under otherwise standard conditions) was found to be only 15 % of that obtained in the presence of pyridoxal phosphate (in the amount of 0.025 μmole). These results indicate that pyridoxal phosphate functions as a cofactor of ornithine δ-transaminase. Since other ω-transaminations also show pyridoxal phosphate dependence (see, for example, NOE AND NICKERSON[11]), it seems likely that such reactions do not differ fundamentally from α-transaminations.

The optimal pH for the action of the transaminase proved to be about 7.4 in 0.1 *M* potassium phosphate. The conditions were standard except for buffer variations, and the pH range used was 6.5 to 8.5. Michaelis constants for the substrates, L-ornithine and α-ketoglutarate, were found to be 2.0 and 1.7 m*M*, respectively; half-maximal enzyme activity was obtained at a pyridoxal phosphate concentration of 0.0017 m*M*. Except for the range of initial substrate or cofactor concentrations employed in these experiments, the test conditions were standard.

That the transaminase is highly specific is indicated by the fact that no appreciable enzyme activity is demonstrable (under otherwise standard conditions) when L-lysine is substituted for L-ornithine or when oxaloacetate, pyruvate, or β-ketoglutarate is substituted for α-ketoglutarate. Any ω-transamination undergone by the lysine could have been detected, since the expected product, α-aminoadipic δ-semialdehyde (like glutamic γ-semialdehyde), gives a color reaction with *o*-aminobenzaldehyde[12].

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Under the test conditions employed, at a concentration of 4 mM, Cu^{++}, Co^{++}, and p-chloromercuribenzoate are inhibitory to the enzymic reaction to the extent of 97, 46, and 91 %, respectively. The pronounced effect of p-chloromercuribenzoate suggests that the transaminase is a "sulfhydryl" enzyme.

In order to test for a possible association of the transaminase with cytoplasmic particles, crude extracts were prepared with mannitol (rather than with sucrose) as a protective agent[13,14], at a final concentration of 0.4 M, and were centrifuged at 100,000 × g for 1 h. When the resulting supernatant liquid and sediment were separately assayed under standard conditions, almost all the transaminase activity was found in the supernatant liquid. The enzyme, therefore, does not appear to be appreciably associated with particles that are sedimentable under the conditions employed, although possible artifacts have not been excluded (*cf.* KOPAC[15]). In view of certain organizational features of the arginine pathway, as revealed by tracer[8] and other[16] studies with *Neurospora*, it must be concluded that ornithine δ-transaminase, despite its apparently "soluble" character, forms part of a closely coordinated biosynthetic system.

This investigation was aided by the Office of Naval Research, Department of the Navy, and by the Atomic Energy Commission, Contract No. AT-(30-1)-1017. The authors are indebted to Dr. D. M. BONNER for laboratory facilities that he generously made available at the Department of Microbiology, Yale University. The constructive interest of Dr. H. J. VOGEL in this investigation is gratefully acknowledged. This article is based on material from the dissertation submitted by R. H. VOGEL to the faculty of the Graduate School of Arts and Science of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June 1958.

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Received May 26th, 1959

Formation of N-phenyl-D-fructosylamine by enzymic transfructosylation from sucrose to aniline*

In enzymic transglycosylation reactions the acceptors for transferred sugar residues are not necessarily restricted to hydroxy compounds like simple alcohols and sugars but other compounds such as inorganic phosphate and carboxylic acids have been shown to be involved[1]. On the other hand the occurrence of N- and S-glycosides in nature suggests, as we have pointed out[2], that amines and thiol compounds may also serve as glycosyl acceptors. These considerations prompted us to look for enzymic transglycosylation reactions in which amines are utilized as acceptors. This paper describes the formation of N-phenyl D-fructosylamine by the enzymic transfructosylation between sucrose and aniline.

In the case of the enzymic formation of N-glycosides of amines special attention must be paid to the fact that spontaneous condensation of free sugars with amines is liable to take place even under physiological conditions[3]. Therefore, in the present experiments the non-enzymic formation of N-glycosides was carefully controlled.

A mixture of sucrose and aniline dissolved in phosphate buffer, pH 7.0, was incubated with a solution of takadiastase. The detection of the reaction products was made by paper chromatography as shown in Fig. 1.

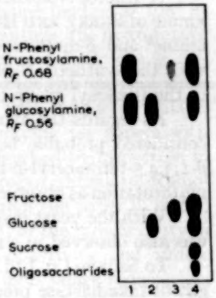

Fig. 1. Paper chromatogram showing enzymic formation of N-phenylfructosylamine. Reaction mixture contained 1 ml 1 *M* sugar, 0.1 ml aniline, 1.9 ml 0.2 *M* phosphate, pH 7.0 and 2 ml of dialyzed solution of 30 % takadiastase. Incubated at 30° for 5 h. Ascending paper chromatography on Toyo No. 50 filter paper; solvent, *n*-butanol–ethanol–water (100 : 15 : 30), spraying reagent, benzidine–trichloroacetic acid for aldose, resorcinol–HCl for ketose, Ehrlich's reagent for aniline. 1. Synthetic N-phenyl fructosylamine and N-phenyl glucosylamine as standard. 2. Aniline, glucose and enzyme. 3. Aniline, fructose and enzyme. 4. Aniline, sucrose and enzyme.

On the chromatogram spots were recognized corresponding to sucrose, glucose, fructose and oligosaccharides arising from sucrose by transfructosylation. Two spots with higher R_F also appeared. The color reaction revealed that the one with R_F 0.68 was composed of fructose and aniline and the other with R_F 0.56 of glucose and aniline. These two spots were identified as those of N-phenylfructosylamine and N-phenylglucosylamine by reference to the R_F values and color reactions of the compounds synthesized according to HONEYMAN and coworkers[4,5]. Controls in which sucrose was substituted for either glucose or fructose showed that under these experimental conditions spontaneous formation of N-phenylglucosylamine was considerable whereas that of N-phenylfructosylamine was almost negligible. The enzymic formation of N-phenylfructosylamine was also observed when tris(hydroxylmethyl)aminomethane of the same pH was used in place of phosphate buffer.

For the isolation of N-phenyl fructosylamine, 95 ml 0.264 *M* sucrose, 5 ml aniline, 50 ml 0.2 *M* phosphate, pH 7.5 and 100 ml dialyzed 30 % takadiastase were incubated at 30°. After 6 h the mixture was heated at 100° for 3 min and filtered. The filtrate was concentrated *in vacuo* to a thin syrup and added to 4 vol. hot ethanol. The ethanolic filtrate was concentrated *in vacuo* until crystals began to appear and placed in a refrigerator to complete crystallization. From the control incubation mixture, in which sucrose was replaced by an equimolar mixture of glucose and fructose, no such crystals were obtained. The product was recrystallized twice from ethanol yielding 249 mg. The substance had m.p. 151° (decomp., uncorr.), unchanged by admixture with synthetic N-phenylfructosylamine, m.p. 151°. $[\alpha]_D^{12}$ —186° → —175° in pyridine (*c*, 0.5). (Found: C, 56.36; H, 6.72; N, 5.54. $C_{12}H_{17}ON_5$ requires C, 56.46; H, 6.71; N, 5.49.) The synthetic sample had $[\alpha]_D^{12}$ —213° → —196° under these conditions. BARRY AND HONEYMAN[5] gave for their synthetic N-phenyl-D-fructosylamine m.p.151°; $[\alpha]_D^{19}$—209° → —164.1° pyridine (*c*, 1). Hydrolysis of the isolated compound with 0.5 *N* HCl at 15° yielded fructose and aniline in equimolar proportion. The infrared spectra of the enzymic product and the synthetic compound were identical.

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* Presented at the 10th Symposium on Enzyme Chemistry of Japan, Sapporo, July 13, 1958.

Apart from a slight disagreement of the specific rotation, the results obtained would indicate that the substance produced by the action of takadiastase upon a mixture of sucrose and aniline was identical with N-phenyl-D-fructosylamine reported by HONEYMAN *et al.*[5].

A fructopyranoside structure has been assigned to the N-phenyl-D-fructosylamine of BARRY AND HONEYMAN[5], as was also the case with *p*-ethoxyphenylfructosylamine[6] and *p*-methoxyphenylfructosylamine[7]. The identity of our enzymic product with the synthetic N-phenylfructosylamine suggests that a change in ring structure of the fructosyl group took place, either enzymically or spontaneously.

As regards the anomer structure of aryl-D-fructosylamines the β-form has been considered probable because of their upward mutarotation and the formation of β-1,3,4,5-tetraacetyl-D-fructose by acetylation followed by hydrolysis[5]. The upward mutarotation as observed with our enzymic product suggests also its β-fructoside form.

With the yeast invertase preparation the formation of N-phenyl fructosylamine was also observed in a similar way.

To Sankyo Co. Ltd. the authors wish to acknowledge with gratitude the generous gift of takadiastase preparation.

The present work was supported in part by a grant from the Ministry of Education.

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Received May 22nd, 1959

Transfer of DNA-glucose from parental to offspring phage T2

Labeled phosphorus, purines, and pyrimidines of DNA are transferred from parental to offspring phage T2 particles with equal efficiency and at least partly in the form of polynucleotides[1]. The DNA of T2 contains glucose attached in glucosidic linkage to the alcoholic group of 5-(hydroxymethyl)cytosine[2]. The question of hereditary transfer of the glucose is of somewhat special significance because one could imagine that removal of the glucose might be prerequisite to replication of the DNA, in which case the parental glucose molecules would not reappear in DNA in the absence of a specific carrier. The following tests show, however, that labeled glucose is transferred with the same efficiency as other DNA constituents.

Phage stocks uniformly labeled with ^{14}C by growth on uniformly labeled invert sugar or fructose were prepared by an "early lysis" method[3]. These stocks showed an absorbancy of about $8 \cdot 10^{-12}$ cm^2 per phage at 260 mμ and contained $2 \cdot 10^{-10}$–$3 \cdot 10^{-10}$ μg C per phage as estimated from the nominal specific radioactivity (0.05 mC/mg C) of the substrate.

The phage preparations (or bacteria infected with them) were analyzed for labeled glucose and adenine as follows. After precipitation of the sample in the cold with trichloroacetic acid, carrier glucose and adenine were added, the DNA was extracted with hot trichloroacetic acid, and the extract was reduced to small volume at 100° (ref. 3). Liberation of glucose and purines was completed by further heating at 100° in 1 *N* HCl for 1 h. After removal of HCl *in vacuo* at room temperature, the hydrolyzate was transferred to paper and subjected to chromatography in two directions in isopropanol[3]. (The isopropanol–HCl solvent is not recommended for this purpose since the acid liberates materials from the paper that give a color with aniline phthalate. This effect was minimized in the present experiments by spraying the papers with ethanol several times during drying.) The purine spots were marked by inspection in u.v. light and the glucose spot by spraying with aniline hydrogen phthalate. Radiocarbon in adenine and glucose was assayed after extraction from the paper in water. Blanks cut from the rims of the spots contained less than 10 % of the radioactivity in the spots and radioautographs showed coincidence of radioactivity and visible material. The recovered adenine contained about 4 % of the total phage radiocarbon and the molar ratio of glucose to adenine fell between 0.32 and 0.49 in numerous measurements, usually close to the value 0.39 expected from other types of analysis[2].

TABLE I

TRANSFER OF GLUCOSE AND ADENINE

Results are shown for a final experiment and, in parentheses, averages for six experiments.

	Phage per bacterium	*[^{14}C]adenine %*	*Molar ratio, labeled glucose:adenine*
Parential phage stock	—	—	0.37
Whole infected culture	—	100	0.35 (0.38)
Early lysis blank	1	4	0.34
Offspring phage particles	70	50	0.35 (0.38)

Abbreviation: DNA, deoxyribonucleic acid.

The transfer measurements were made by infecting cells in a culture medium containing unlabeled glucose as described previously[3] and included analyses of three materials: a sample of the whole infected culture taken after removal of unadsorbed input phage by centrifugation; a blank consisting of u.v.-inactivated phage particles added to a sample of the culture lysed at 13 min after infection and then reisolated; and a phage progeny isolated in the same way after lysis at 60 min. Recovery of the phage progeny (70 to 150 per bacterium) during purification, measured by plaque titration, was 33 % to 49 %, and transfer of adenine, corrected for these losses, measured 50 % to 65 %, in six experiments. The ratio of labeled glucose to labeled adenine was the same in all of the materials analyzed, as shown in Table I.

This work was aided by grant C 2158 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

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Received June 3rd, 1959

A requirement for pyrimidines in the first few minutes of T2 bacteriophage development

Phage DNA synthesis occurs about 6 min after T2 infection in *Escherichia coli*[1]. The present experiments were designed to test whether a supply of pyrimidine was necessary prior to this DNA synthesis, for subsequent normal phage development in the infected bacteria.

In order to permit analysis of nucleic acid synthesis by ^{32}P incorporation all experiments were carried out in a synthetic low-phosphate, glycerol–lactate medium[2]. The techniques were in general those described in a previous communication[3].

Blockage of pyrimidine supply within the cell was brought about by the use of the uracil analog 6-azauracil*. This compound is known to function in uninfected bacteria through inhibition of orotidylic decarboxylase, an enzyme of pyrimidine synthesis[4]. Apparently 6-azauracil causes a rapid and almost complete suppression of pyrimidine synthesis since 150 μg/ml 6-azauracil can be completely reversed by as little as 1 μg/ml of uracil. Thus, the available pyrimidine supply in uninfected *E. coli* must rapidly drop to a very low level when 6-azauracil is added; and nucleic acid synthesis, as measured by $H_3{}^{32}PO_4$ incorporation into material insoluble in cold trichloracetic acid and alcohol is reduced at least 86 % within 2 min of the addition of the analog. Protein and β-galactosidase synthesis are correspondingly reduced only after a 10-min lag.

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

* 6-Azauracil was a gift from Dr. A. D. WELCH.

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When 6-azauracil is added at the time of T2 infection of *E. coli* the same general picture of inhibition of nucleic acid synthesis is observed. Analysis of 12-min or 16-min samples (either by a colorimetric method for DNA (Table I) or by content of 5-hydroxymethylcytosine) showed an 80 % inhibition of DNA synthesis in a culture to which 300 µg/ml 6-azauracil was added compared to a control in which the analog was absent. Protein synthesis in infected cultures (as measured by $[^{14}C]$leucine uptake) was also reduced about 80 % in the presence of 6-azauracil.

TABLE I

INHIBITION BY 6-AZAURACIL OF PHAGE PRODUCTION AND DNA SYNTHESIS

The bacteria were infected for 5 min in buffer + 0.002 *M* CN^- with 10 T2 phage/bacterium. Over 99 % of the bacteria were infected. The culture was centrifuged and resuspended in the original growth medium; then 300 µg/ml 6-azauracil was added to aliquots at various times. Samples for DNA analysis were taken at 17 min, and samples for intracellular phage (lysed with chloroform before planting) were taken at 30 min.

Time of addition (min)	*Phage/bacterium*	*% increase of DNA*
0	0.0	12
3	1.1	28
6	8.0	64
not added	4.7	106

Three kinds of experiments to be described below indicate that it is a very early step in T2 development which is inhibited by 6-azauracil and that consequently the presence of pyrimidines must be essential at these early times.

First, in order to discover how much phage development occurred in the presence of 6-azauracil, 300 µg/ml of the compound was added to the culture at 0 min (the time of infection), at 3 min and at 6 min. When the analog was added at 0 min, no intracellular phage as subsequently produced. Addition at 3 min after infection allowed some phage development, and when addition was made at 6 min no inhibition occurred (Table I). Some stage in the development of bacteriophage which occurs before 3 min is apparently affected by 6-azauracil. This assumption is borne out by the following observation. When inhibition of a culture to which 6-azauracil is added at the time of infection is reversed at 20 min by the addition of 3 µg/ml uracil no intracellular phage appear until after the normal lag period of an additional 12 min. If any of the normal processes in phage development had been carried out efficiently in the presence of 6-azauracil during the first 1 or 2 min of infection, this would have been reflected in a lag period after reversal which would be shorter than 12 min. (In the case of proflavin, which is known to inhibit a late stage of phage development, phage appear at 1 min after reversal[5]).

A third piece of evidence which indicates an early effect of 6-azauracil during phage production is found in studies of resistance to u.v. light of the plaque-forming ability of the infected bacteria. The gradual slow increase in resistance to u.v. which is characteristicly complete in the first 6 min after T2 infection[6] requires 12 min for development in the presence of 6-azauracil (Fig. 1). The subsequent rapid and large increase in resistance which takes place shortly after 6 min in the control culture does not occur within 30 min in the 6-azauracil-inhibited culture. The fact that 6-azauracil does not permit development of u.v. resistance equal to that of a control at 6 min indicates a requirement for pyrimidines prior to 6 min.

The experimental results thus point to the necessity for a pyrimidine supply during the early minutes of T2 development. This might have been anticipated since infected bacteria must synthesize essential proteins in the first few minutes after infection[7], and in uninfected *E. coli* protein synthesis requires nucleic acid precursors[8].

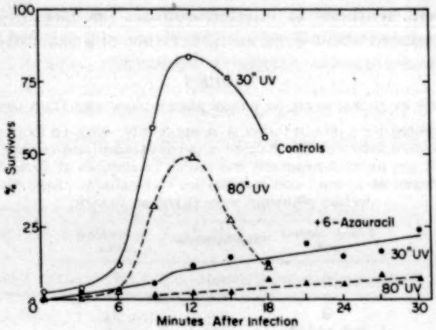

Fig. 1. Changes in resistance to u.v. light of T2-infected *E. coli*. The bacteria were infected for 5 min at a multiplicity of 0.02 in buffer + 0.002 *M* CN^-, and were resuspended in their original medium to which had been added 300 µg/ml 6-azauracil (+ 10 µg/ml uracil for the control only). Survivors to 30 or 80 sec u.v. were measured at intervals after resuspension. At 31 min the control and inhibited cultures, respectively, gave yields of 9 and less than 0.01 intracellular phage per original infectious center.

The observation that an external supply of purines is not essential for early stages of phage development[7] does not rule out synthesis of nucleic acids as an obligatory early step in T2 reproduction since internal reserves of purines could delay expression of the purine requirement. Indeed, RNA with a base composition different from that of the RNA of the uninfected host cell has been found in T2-infected *E. coli*[9]. This RNA is formed at the beginning of infection[10]. It is quite possible that a pyrimidine shortage blocks the formation of this new kind of RNA which is in turn involved in the synthesis of new kinds of proteins.

This work was aided from the University of California Cancer Research Funds, and by a grant from the Rockefeller Foundation.

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Received May 19th, 1959

Purification of pyrimidine bases from hydrolyzed pyrimidine nucleotides

In the course of studies on the incorporation of injected [^{14}C]cytidine into rat-liver nucleic acids[1] it was necessary to hydrolyze isolated pyrimidine nucleotides to free bases and measure radioactivity to determine if intact incorporation of the sugar and base moieties of the injected cytidine into RNA occurred. Due to relatively low incorporation of the radioactive material, a high degree of purity of the free bases was essential for accurate interpretation of the results. The presence of caramelized material in hydrolysates of pyrimidine nucleotides presented some difficulties in efforts to obtain the desired purity by conventional chromatographic techniques. Ratios of absorbancy measurements at 250/260, 280/260, and 290/260 mμ taken on solutions of bases at pH 2.0, 7.0, and 12.0 and measurements of specific activity following various purification procedures were used as criteria for purity of the isolated pyrimidine bases.

A number of hydrolysis procedures were tested with cytidylic and uridylic acids isolated from liver ribonucleic acid but in all cases caramelized material appeared on paper chromatograms as visible brown spots (blue fluorescence under u.v. light) with R_F values similar to those of cytosine and uracil. Recourse to other chromatographic solvent systems did not solve the problem. The difficulty was circumvented in the following manner: Hydrolysis with conc. formic acid, according to the procedure of VISCHER AND CHARGAFF[2], was used because it gave a higher yield of free base than the other methods tried. Water-saturated *n*-butanol[3] was used as the solvent system for paper chromatography. After hydrolysis of cytidylic acid, the mixture was evaporated to dryness, taken up in a drop of water and applied to a 2.5 × 60 cm strip chromatogram and developed in the butanol–water solvent system. Compounds identified on the chromatogram were unhydrolyzed cytidylic acid which remained at the base line in the above mentioned chromatographic system, cytidine (R_F, 0.15), uridine (R_F, 0.18), cytosine (R_F, 0.26), and uracil (R_F, 0.36). After development, the section of the chromatogram just below the uridine spot was cut off and eluted with distilled water. The solution was evaporated to dryness under vacuum and then taken up in 1 ml 2 *N* HCl. This acid solution was adsorbed on a 1 × 15 cm Dowex 50 (200–400 mesh, 8 × cross linked) column, which had previously been washed with 500 ml 2 *N* HCl. Elution was carried out with 2 *N* acid and 3-ml fractions were collected. Uracil and caramelized material came off in the first few fractions, but after 60–70 ml of acid passed through, cytosine came off the column in 2 to 4 fractions. Absorbancy ratios were in agreement with published values indicating a high purity for the cytosine fraction.

The above system was not satisfactory for purification of uracil from hydrolyzed uridylic acid. A search of the literature revealed that paraldehyde formed a precipitate with caramel[4]. Paraldehyde additions prior to chromatography were tried and found to work very well. The following procedure was employed: After hydrolysis, formic acid was removed under vacuum. Then 0.05 ml water, 0.25 ml paraldehyde, and enough ethanol to make the two phases miscible (0.1–0.2 ml) were added, and the mixture allowed to stand overnight (about 16 h) in a stoppered test tube. The mixture was evaporated to about one drop and then applied to a chromatogram. On develop-

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Abbreviation: RNA, ribonucleic acid.

ment in the butanol–water system, the caramelized material remained at the base line. The spot containing uracil was eluted with distilled water and absorbancy ratios agreed very well with published values. If the solution of uracil was made slightly alkaline and passed though a short column (1 × 5 cm) of Dowex 1 Cl^- form, the slight contaminants picked up from the paper were removed and absorbancy ratios were in exact agreement with literature values. Some of the results obtained by this procedure for uracil isolated from the RNA obtained from four different animals are listed in Table I. Before paraldehyde treatment, the specific activity of the bases varied with each operation for purification. After paraldehyde treatment, the specific activity remained constant through several repetitions of the purifications procedure.

TABLE I

ABSORBANCY RATIOS AND SPECIFIC ACTIVITY OF URACIL* BEFORE AND AFTER TREATMENT WITH PARALDEHYDE

	Before paraldehyde treatment				*After paraldehyde treatment*			
	Absorbancy ratios pH 7.0			*Specific activity counts/min/µatom Carbon*	*Absorbancy ratios pH 7.0*			*Specific activity counts/min/µatom Carbon*
	250/260	*280/260*	*290/260*		*250/260*	*280/260*	*290/260*	
Literature value	0.84	0.175	0.010	—	0.84	0.174	0.010	—
Sample 1	0.79	0.22	0.020	8	0.84	0.175	0.010	19
Sample 2	0.92	0.24	0.008	5	0.84	0.170	0.010	19
Sample 3	0.94	0.23	0.020	6	0.84	0.180	0.010	22
Sample 4	1.02	0.64	0.500	30	0.83	0.180	0.006	40

* Uracil was obtained by hydrolysis of uridylic acid of rat liver RNA isolated by the classical Schmidt-Thannhauser procedure and ion-exchange chromatography. The nucleotides were of very high purity based on absorbancy ratios.

The described technique may be very useful in other studies in which a high state of purity of pyrimidine bases is required.

This work was supported in part by the Division of Biology and Medicine, U.S. Atomic Energy Comission. Journal Paper No. 180, American Meat Institute Foundation.

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Received June 3rd, 1959

Di- et triphosphopyridine nucleotides (DPN et TPN) de christallins de bovidés jeunes et âgés

Dans une série de recherches antérieures, nous avons mis en évidence une réduction des diverses activités déshydrogénasiques[1-3] et des phosphorylations oxydatives[4] du cristallin au cours du vieillissement. Une des expressions de ces phénomènes était la diminution des nucléosides triphosphates au niveau de cristallins âgés ou même au niveau des fibres relativement âgées de cristallins jeunes[5,6]. Pour préciser les conséquences éventuelles d'une baisse des activités enzymatiques constatée à l'aide de mesures *in vitro* et de ce fait toujours sujette à critique, il nous a paru intéressant d'évaluer les rapports DPNH/DPN+ et TPNH/TPN+. On sait que le premier est dominé par les activités déshydrogénasiques[7] et les phosphorylations oxydatives[8-10] alors que le second serait contrôlé par certaines activités déshydrogénasiques d'une part[11], les biosynthèses cellulaires utilisant le TPNH d'autre part[12].

Nos essais ont porté sur des cristallins de bovidés jeunes et âgés, prélevés sur l'animal dans un délai de 15 à 30 sec après le sacrifice par coup de pistolet, et immergés immédiatement dans de l'azote liquide. Les déterminations se font d'après la méthode de HOLZER et coll.[13] par un ensemble de 4 réactions enzymatiques employant pour le DPNH et le TPNH respectivement l'α-glycérophosphate déshydrogénase et l'acide glutamique déshydrogénase, pour le DPN et le TPN l'alcool déshydrogénase et le Zwischen ferment avec du glucose-6-phosphate. Pour chaque animal, un cristallin est extrait à chaud par le NaOH en vue des dosages du DPNH et TPNH et l'autre cristallin homologue par l'acide à froid pour les déterminations du DPN et TPN.

Les résultats de nos essais sont consignés dans le Tableau I.

TABLEAU I

LES VALEURS SONT EXPRIMÉES EN mg POUR 100 g DE POIDS FRAIS

DPNH *mg*	*DPN* *mg*	$\frac{DPNH}{DPN}$	*DPNH + DPN* *mg*	*TPNH* *mg*	*TPN* *mg*
		Bovidés jeunes			
0.8	13.7	0.06	14.5	1.1	< 0.3
0.8	12.9	0.06	13.7	1.9	< 0.3
1.0	17.9	0.05	18.9	2.0	< 0.3
1.0	16.2	0.06	17.2	1.9	< 0.3
1.0	18.3	0.05	19.3	2.0	< 0.3
Moyenne					
0.9	15.8		16.7	1.7	
± 0.1	± 2.4		± 2.5	± 0.4	
		Bovidés âgés			
0.7	7.5	0.09	8.2	4.3	< 0.3
0.4	11.8	0.03	12.2	3.3	< 0.3
0.5	9.3	0.05	9.8	3.7	< 0.3
0.8	10.1	0.05	10.9	4.8	< 0.3
Moyenne					
0.6	9.6		10.2	4.0	
± 0.2	± 1.8		± 1.7	± 0.7	

---

[*] Abbréviations: DPN+, DPNH, diphosphopyridine nucleotide oxydé et réduit; TPN+, TPNH, triphosphopyridine nucleotide oxydé et réduit.

Il en ressort que la somme DPN + DPNH est nettement inférieure dans le cristallin de bovidés âgés; les valeurs les plus élevées de ces derniers sont au-dessous des valeurs les plus faibles de cristallins jeunes. Quant au rapport DPNH/DPN il n'accuse pas de différence significative. Il apparaîtrait donc que la diminution à la fois du DPN oxydé et réduit est rigoureusement parallèle à la baisse des activités déshydrogénasiques d'une part, des phosphorylations oxydatives d'autre part, laissant ainsi un rapport DPNH/DPN inchangé. Notons que les seuls chiffres de DPN et DPNH que nous avons relevés dans la littérature[14] et qui remontent à 1939 concernent des bovidés d'un âge non déterminé. Les valeurs de la somme DPN + DPNH dans ce cas se situent dans la zone des nôtres pour les animaux adultes alors que le rapport DPNH/DPN est bien plus élevé que dans nos essais. Or nous avons également trouvé des valeurs plus fortes pour le rapport DPNH/DPN quand les délais de prélèvement des cristallins après le sacrifice sont plus longs et si l'on refroidit les cristallins par la neige carbonique au lieu de la faire par l'air liquide.

Les teneurs de TPN et de TPNH du cristallin dont nous n'avons trouvé aucune indication dans la littérature soulèvent un problème curieux. Si le rapport TPNH/TPN est semblable à celui trouvé pour d'autres tissus[15], on est par contre frappé par l'augmentation de ces pyridines nucléotides dans le cristallin âgé alors que nombre d'indices témoigne d'une réduction de l'activité métabolique. L'explication de ce phénomène appelle évidemment d'autres recherches mais nous pensons qu'il est permis dès à présent de formuler une hypothèse de travail. L'accroissement du TPNH peut être dû à un phénomène d'adaptation. Les quantités d'accepteurs de l'hydrogène du TPN qui sont avant tout des métabolites de la biosynthèse étant réduites, les cellules répondent par un accroissement de la biosynthèse du pyridine nucléotide, ce qui rend possible le déroulement du cycle oxydatif direct, moins touché par le vieillissement que la glycolyse.

Nous remercions le Professeur H. Holzer, Directeur du Physiologisch-Chemische Institüt de l'Université de Freiburg (Allemagne) qui a bien voulu nous accueillir dans son laboratoire pour nous initier à sa méthode de dosage de pyridine nucléotides.

Travail effectué avec l'aide technique de Madame A. M. Ruch.

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Reçu le 6 juin 1959

The presence of adenosine diphosphoribose and adenosine triphosphoribose in acid extracts of certain animal tissues

The oxidized forms of DPN and TPN are enzymically cleaved at the nicotinamide-riboside bond by DPNase whereas the reduced form of DPN (and presumably TPN) is not affected by this enzyme[1]. In the oxidized state these nucleotides are relatively stable in acid solution, but, at pH values of 4 or lower, DPNH is rapidly destroyed even at room temperature[2]. The products of this reaction have not been positively identified but it has been demonstrated that acid treatment of DPNH can give rise to ADPR and a nicotinamide derivative[3]. It has been further suggested[4] that the compounds designated as Ad and ADPX by BRUMM *et al.*[5] are the acid-decomposition products of DPNH and TPNH. HANSEN *et al.*[6] have identified a nucleotide in liver (ADPR) which corresponds to the Ad compound[7].

During an investigation of the acid-soluble nucleotides of fish, chicken and rat liver[8] using the technique of HURLBERT *et al.*[9], a qualitatively similar pattern of nucleotide elution was obtained with all three species. The same technique, applied to rat mammary gland (Fig. 1), also produced a qualitatively similar pattern. In each case, the nucleotides found in the two areas designated as G and O_1 were concentrated[10] and analyzed (Table I). In all cases the nucleotides isolated had an absorption spectra corresponding to an adenosine derivative. Chemical analysis showed that the major constituent found in peak G had 2 moles of ribose and 2 of phosphorus/mole of base whereas the major constituent found in peak O_1 had 2 moles of ribose and 3 moles of phosphorus/mole of base. These data are consistent with the designated formulas ADPR and ATPR for these two compounds. To demonstrate that these compounds could have arisen from DPNH and TPNH, an additional series of experiments were carried out as follows: (a) 8 mg DPNH (Sigma) were dissolved in water, placed on a 25 × 1 cm resin bed and subjected to ion-exchange chromatography as described by HURLBERT *et al.*[9]. Two nucleotides were eluted from the column. The first nucleotide, having a maximum absorbance at 260 mμ, was not analyzed but it corresponded to the elution position occupied by DPN. The second nucleotide was analyzed and proved to be ADPR (Table I). (b) 8 mg DPNH were dissolved in 10 ml 0.6 *N* $HClO_4$ and

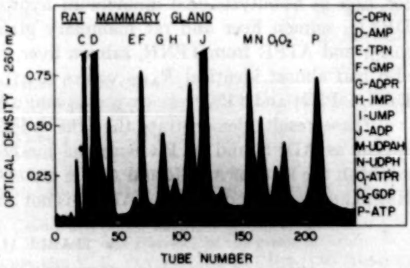

Fig. 1. Separation of the acid-soluble nucleotides of rat mammary gland[9] on a 1.8 × 40 cm resin bed. A 500-ml mixing flask was used and 15-ml fractions were collected[8]. The nucleotides listed with the graph are the major constituents found in the designated areas. Identification was made on the basis of absorption spectra, phosphorus and ribose analysis, and chromatographic behavior (unpublished studies).

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; ADPR, adenosine diphosphoribose; ATPR, adenosine triphosphoribose.

TABLE I

ANALYSIS OF ADPR AND ATPR

Source	ADPR (G)			ATPR (O_1)		
	*Base**	*Ribose*	*PO_4*	*Base**	*Ribose*	*PO_4*
DPNH	1.03	2.30	2.00	—	—	—
TPNH	—	—	—	1.04	2.10	3.00
Salmon liver	1.10	2.25	2.00	1.11	2.21	3.00
Rat liver	—	—	—	1.15	1.92	3.00
Rat mammary gland	1.02	2.13	2.00	1.10	2.31	3.00
Chicken liver	1.00	1.98	2.00	1.26	2.02	3.00

* Determined by absorbance at 260 mµ. Values given in the table have been corrected so that phosphorus appears as a whole number.

left at room temperature for 30 min. The solution was then neutralized, left in an ice bath 12 h, filtered, and treated as in (a). (This acid treatment was carried out to simulate the conditions of tissue extraction.) (c) 11 mg DPN (Sigma) were treated as in (a). (d) 11 mg DPN were treated as in (b). (e) 50 mg TPN (Sigma) were partially reduced[2] and subjected to ion-exchange chromatography without further treatment. Three nucleotides, corresponding to the elution positions of areas E, J, and O_1 (Fig. 1) were obtained. The third nucleotide (15.3 µmoles) was analyzed and determined to be ATPR (Table I). The second nucleotide was not analyzed.

The data from these experiments are summarized in Table II and a composite drawing of the nucleotide elution pattern is illustrated in Fig. 2.

Further to confirm that ADPR and ATPR from tissue and from DPNH and TPNH are identical, the samples were subjected to descending paper chromatography for 27 h in isobutyric acid–ammonium hydroxide–water (66 : 1 : 33)[11]. ADPR from DPNH, salmon liver and rat mammary gland had almost identical R_{AMP} values (0.65) and ATPR from TPNH, salmon liver, rat mammary gland and chicken liver also had almost identical R_{AMP} values (0.41). The corresponding R_{AMP} values for DPN, DPNH and TPN were 0.93, 0.94 and 0.62 respectively.

These results demonstrate that the reduced pyridine nucleotides of tissue will appear as ADPR and ATPR with the methods employed. However, they do not establish the level of ADPR and ATPR present *in vivo*. Recent work by PREISS AND HANDLER[12] would indicate that ADPR is not a necessary precursor for DPN synthesis

TABLE II

AMOUNT OF NUCLEOTIDES RECOVERED AFTER COLUMN CHROMATOGRAPHY

Starting material	*Expt.*	*Pretreatment**	*µmoles nucleotide recovered*			
			DPN	*TPN*	*ADPR*	*ATPR*
DPNH	(a)	None	1.6		11.2	
	(b)	$HClO_4$	0.6		10.6	
DPN	(c)	None	13.2		0.2	
	(d)	$HClO_4$	9.8		1.8	
TPN	(e)	Partially reduced		32.5		15.3

* Described in detail in the text.

but the possibility exists that ADPR is present *in vivo* as a degradation product by virtue of DPNase activity. This would allow for an orderly turnover of DPN without necessitating a reversal of the synthetic mechanism.

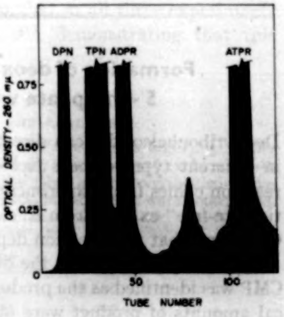

Fig. 2. Composite diagram of the nucleotide separation obtained in Expts. (a), (c) and (e). Ion-exchange chromatography was carried out as described by HURLBERT *et al.*[9] using a 1 × 25 cm column and a 300-ml mixing flask. The column was eluted with 500-ml fractions and aliquots of 10 ml were collected with a Gilson Fraction Collector.

Results presented here confirm the findings of BURTON AND KAPLAN[13] and CHAYKIN *et al.*[3] that ADPR can be an acid-decomposition product of DPNH. Preliminary results (unpublished) obtained in this laboratory indicate that when the C-D area of tissue-nucleotide extracts (Fig. 1) is rechromatographed using the ammonium formate system[9], a compound is obtained which exhibits properties similar to the nicotinamide derivative from DPNH (*i.e.* it has a maximum absorbance at approximately 290 mμ which disappears on standing in acid solution[2]). The significance of this finding is being investigated.

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Received May 2nd, 1959

Preliminary Notes

Formation of deoxycytidine 5′-phosphate from cytidine 5′-phosphate with enzymes from *Escherichia coli*

Deoxyribonucleotides can directly be formed through a reduction of ribonucleotides in different types of cells (see *e.g.* ref. 1). Most of the evidence for such a type of reaction comes from experiments with intact cells. We have now found that a "particulate-free" extract from *E. coli* B carries out the transformation of CMP to deoxy-CMP, and that this reaction depends on the presence of ATP, Mg^{++} and TPNH.

The requirements for the overall reaction are demonstrated in Table I. Deoxy-CMP was identified as the product of the reaction by the following criteria: (1) identical amounts of product were obtained with either ^3H- or ^{32}P-labelled CMP; (2) on paper chromatography[1] the product obtained from either ^3H- or ^{32}P-labelled CMP moved as deoxy-CMP; (3) after dephosphorylation with prostatic phosphatase the product obtained from [^3H]CMP behaved as deoxycytidine on Dowex-50[1] or on paper chromatography.

A strong stimulation of deoxy-CMP formation with ATP + Mg^{++} appears directly with the crude extract. The dependence on TPNH, however, emerges first after treatment of the enzyme with Dowex-2. DPNH stimulated much less.

Negligable amounts of deoxy-CMP (and deoxycytidine) were formed from cytidine 2′(3′)-phosphate or cytidine.

Table II demonstrates results of three experiments during which incubation was

TABLE I

REQUIREMENTS FOR THE FORMATION OF DEOXY-CMP

The complete system contained: 0.05 µmole of ^3H- or ^{32}P-labelled substrate, 1 µmole ATP, 5 µmoles $MgCl_2$, 10 µmoles Tris buffer, pH 7.4. Incubation for 10 min at 37° with 3.4 mg enzyme, final vol. 0.25 ml. The reaction was stopped with 1 ml 1 *M* $HClO_4$. After centrifugation the supernatant was boiled for 10 min, and CMP and deoxy-CMP were separated on Dowex 50[1]. The amounts of deoxy compounds formed were calculated from the radioactivity.

	Radioactive substrate	*mµmole deoxy-CMP formed*
Complete system	[^3H]CMP	0.55
Complete system	[^{32}P]CMP	0.53
Minus ATP and Mg^{++}	[^{32}P]CMP	0.03
Complete system	[^{32}P]cytidine 2′(3′)-phosphate	0.01
Complete system	[^3H]cytidine	0.04
Complete system*	[^{32}P]CMP	0.04
Complete system* + TPNH (30 mµmoles)	[^{32}P]CMP	0.35
Complete system* + DPNH (30 mµmoles)	[^{32}P]CMP	0.10

* Dowex-2-treated enzyme.

Abbreviations: CMP, CDP and CTP, cytidine 5′-mono-, di- and triphosphate, respectively; ATP, adenosine 5′-triphosphate; DPNH and TPNH, reduced di- and triphosphopyridine nucleotide, respectively; Tris, tris(hydroxymethyl)aminomethane.

carried out in the presence of large amounts of non-labelled deoxy-CMP, deoxy-CDP and deoxy-CTP, respectively. In each case the specific activities of the three deoxynucleotides were determined at different time intervals. Only one early-time point is given for each experiment in Table II. It is evident that in all three experiments the highest specific activity was found in deoxy-CDP, demonstrating that this nucleotide was the first product of the reaction.

TABLE II

DEOXY-CDP AS THE FIRST PRODUCT OF THE REACTION

[^{32}P]CMP (1.7 μmoles, $23 \cdot 10^6$ counts/min/μmole), 10 μmoles ATP, 50 μmoles $MgCl_2$, 3 μmoles deoxy-nucleotide and 31 mg enzyme, pH 7.4, were incubated for 4 min at 37° (vol., 2.5 ml). Mono-, di- and triphosphates were separated on Dowex-2, the ribo- and deoxyribonucleotides within each group were then separated on Dowex-50 and their specific activities were determined.

Deoxynucleotide added at start of incubation	*Connts/min/μmole after 4 min*		
	deoxy-CMP	*deoxy-CDP*	*deoxy-CTP*
deoxy-CMP	13,000	77,000	75,000
deoxy-CDP	9,000	27,000	14,000
deoxy-CTP	5,000	20,000	11,000

These experiments thus lead us to the following formulation of the reaction sequence during the reduction of CMP to deoxy-CMP:

$$\text{CMP} \xrightarrow{\text{ATP, Mg}^{++}} \text{CDP} \xrightarrow{\text{TPNH}} \text{deoxy-CDP} \longrightarrow \text{deoxy-CMP}.$$

With CDP as substrate the formation of deoxy compounds was still strongly stimulated by ATP and Mg^{++}. This might indicate that the actual reduction step involves a more complicated course than that shown in the above scheme and that it might involve the synthesis of an intermediate which requires ATP and Mg^{++}.

Our results are not in agreement with those reported earlier by GROSSMAN AND HAWKINS[2]. It was reported that deoxyribosyl compounds could be formed in a rather unspecific way from ribonucleotides or ribonucleosides with an extract of *Salmonella typhimurium*. The reaction did not require ATP or TPNH, but was strongly stimulated by dithiopropanol. Besides, considerably larger amounts of deoxyribosyl compounds were formed than in our experiments.

Using the techniques reported by GROSSMAN AND HAWKINS we have to a large extent been able to confirm the results of these authors with *S. typhimurium*. However, when comparing the amounts of deoxyribosides formed as measured by deoxyribose analysis with the results of isotopic assays, it became evident that only a very small fraction of the deoxyribosyl compounds conceivably could have arisen through a direct reduction of ribosyl compounds. Instead, it seems likely that the results with *S. typhimurium* can be best explained on the basis of the action of deoxyribosyl-transferring enzymes.

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Received November 26th, 1959

Labelling of microsomal proteins with radioactive amino acids

Recent investigations have shown that labelled amino acids are rapidly incorporated into the microsomal ribonucleoprotein and then transferred to the other protein fractions of the cell. However, the role of any fraction other than RNP in the microsome has received relatively little attention. COHN AND BUTLER[1], who fractionated the microsomal proteins into smaller fractions by a chemical method, have revealed that all the microsomal proteins, except RNP, were labelled simultaneously. The author's attempts to subdivide the deoxycholate-soluble fraction of the microsome by starch zone electrophoresis had similar result. However, when an immunological method was applied to the fractionation of proteins, a heterogeneous labelling of the DC-soluble fraction was demonstrated.

Albumin and globulin fractions were separated from the serum of a rat by electrophoretic separation, and anti-sera for albumin and globulin were prepared by injecting into albino rabbits six times at 3-day intervals with increasing doses. Microsomal and supernatant fractions were prepared from rat liver by a conventional cell-fractionation technique[2], and the microsomal fraction was separated into a DC-soluble and a DC-insoluble fraction[3]. RNP was purified from the DC-insoluble fraction[4]. When anti-serum for serum albumin or globulin was added to the fractions and incubated for 2 h at 37°, precipitation was obtained from the DC-soluble and the supernatant fractions in certain amounts, but not from the RNP. The precipitate was separated, washed, and dissolved in 1 *N* NaOH, and the quantity of protein was determined with Folin's reagent[5]. The quantity of the material precipitated by anti-serum was estimated from a standard curve. The percentage of the material precipitated by anti-albumin serum to the original fraction was approximately 4 % in the DC-soluble and 8 % in the supernatant fraction, and that by anti-globulin serum was approximately 5 % in the DC-soluble and 7 % in the supernatant fraction. When both antisera were added together to the DC-soluble or the supernatant fraction, the amount of the precipitated portion was almost the sum of the separate experiments.

At 3, 8, and 20 min after the intravenous injection of 0.03 mC radioactive amino acids (an equivalent mixture of DL-[^{14}C]leucine, DL-[^{14}C]glutamic acid, and DL-[^{14}C]-lysine) per group of four rats, the livers were quickly removed, homogenized, fractionated and the specific activity of each fraction determined. The results are summarized in Table I. A marked difference was observed between the precipitated and non-precipitated portions. About 40–50 % of the counts of the original fractions were recovered in the material precipitated by both anti-sera. The specific activity (counts/min/mg protein) of the precipitated material was about ten and four times as high as that of the non-precipitated portion of the DC-soluble and the supernatant fraction, respectively, while that of anti-albumin and anti-globulin sera was at almost the same level.

The results indicate not only the heterogeneous labelling of microsomal proteins other than RNP but also the course of serum-protein synthesis. From Table I, materials precipitated by anti-serum can be arranged in decreasing order of specific activity as follows: DC-soluble fraction > supernatant fraction > serum. If it is assumed that the material precipitated by anti-serum in the DC-soluble fraction is a precursor of serum protein as suggested by PETERS[6], the amino acids incorporated

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Abbreviations: RNP, ribonucleoprotein; DC, deoxycholate.

TABLE I

LABELLING OF PROTEINS IN FRACTIONS OF RAT-LIVER CYTOPLASM BY RADIOACTIVE AMINO ACIDS

	Microsomal fractions					*Supernatant fraction*			*Serum*	
	RNP		*DC-soluble*							
Time after injection (min)	3	20	3	8	20	3	8	20	3	20
	Specific activity (counts/min/mg protein)									
Whole fraction	91	76	18	67	179	7	34	115	0	16
Material precipitated by										
anti-albumin serum	(no		92	367	927	15	86	294	0	18
anti-globulin serum	precipitation)		87	346	901	17	93	316	0	15
Non-precipitated portion*			8	36	98	4	24	73	—	—

* The specific activity of the non-precipitated portion was estimated from the difference in counts between the portions precipitated by both anti-sera and the whole fraction.

initially into the RNP will be transformed into the precursor protein of the DC-soluble fraction, move to the supernatant fluid, and finally enter the circulating system.

Another speculation is that the microsome synthesizes two kinds of proteins, the structural proteins of the cell and the serum proteins. The fact that the specific activity is higher in the precursor proteins of serum than in the non-precipitated portion shows that the rate of serum-protein synthesis is much higher than that of structural proteins in adult rats. MILLER *et al.*[7] have demonstrated in their study of isolated rat liver perfused with oxygenated blood containing radioactive amino acid that the liver synthesizes practically almost all the plasma proteins, as well as liver proteins, and estimated that the turnover rate of circulating plasma is about three times as high as that of liver proteins. These results may support the speculation mentioned above. The author will submit more detailed information on these studies later.

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Received November 27th, 1959

Biochim. Biophys. Acta, 37 (1960) 556–557

A soluble DPNH dehydrogenase from heart-muscle particles

Two well documented DPNH-dehydrogenating enzymes have been isolated by heating heart-muscle particles in aqueous alcohol. They are the diaphorase of STRAUB[1] and the DPNH-cytochrome *c* reductase of MAHLER and co-workers[2]. Both enzymes can use a number of artificial electron acceptors whereas only Mahler's enzyme is also active with cytochrome *c*. Pertinent facts[3] suggest that Mahler's enzyme may be a

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; MB, methylene blue.

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modified form of the "true" DPNH dehydrogenase *in situ*. Recently Massey has found that practically pure diaphorase prepared by his improved method possesses lipoic dehydrogenase activity[4]. In fact, he has demonstrated that the enzyme is a component of the α-ketoglutaric dehydrogenase complex[5]. Independently, Searls and Sanadi[6] have isolated diaphorase to the electrophoretically pure form from the purified dehydrogenase complex. This discovery raises the salient question whether diaphorase is the "true" unmodified DPNH dehydrogenase which can perform dual functions as a component of α-ketoglutaric dehydrogenase complex and as a member of the main respiratory chain.

In the search for DPNH dehydrogenase in heart particles we purified a soluble enzyme which was able to use DCIP and ferricyanide as electron acceptor but was practically inactive toward cytochrome *c* and lipoic acid.

Ball[7], Ambe[8] and co-workers have reported that snake venom can destroy the DPNH oxidase activity of heart-muscle particles. We confirmed their findings and observed that the DPNH oxidase activity[*] of the beef heart-muscle preparation made according to Keilin and Hartree[13] was rapidly inactivated by digestion with boiled or unboiled snake venom. The inactivation was apparently due to the action of lecithinase A which is heat stable. A very large temperature coefficient of the inactivation was found. The half time was about 20 min at 37° and 240 min at 25° in the system containing in 1 ml 0.1 M phosphate buffer, pH 7.4, 10^{-4} M $CaCl_2$, heart-muscle preparation equivalent to 10 mg dry organic solid, and 3.5 μg boiled Naja-Naja venom. Under these conditions a negligible activity of soluble DPNH dehydrogenase tested with DCIP emerged. However, the liberation of a soluble DPNH dehydrogenase was observed with high concentrations of venom and at lower pH. More than 70 % of the dehydrogenase was solubilized by incubating the system mentioned above for 2 h at 37° in the presence of 32 μg boiled venom/mg dry weight of the heart-muscle preparation at pH 6.5. About 50-fold increase in specific activity with approximately 15 % yield was obtained by fractionation of the supernatant fraction from the venom digest with ammonium sulfate (0.30–0.50 saturation), followed by differential adsorption on calcium phosphate gel (adsorbed from 0.01 M phosphate, pH 7.4 and eluted with 0.2 M phosphate).

The enzyme was fluorescent. In contrast to diaphorase[10], its fluorescence disappeared immediately upon the addition of DPNH.

The enzyme was not sedimented by centrifuging the solution at 140,000 × *g* for 1 h. It was not stable even at low temperatures; for example, about 25 % of the activity was lost upon storage at —15° for 20 h. This is reminiscent of the soluble succinic dehydrogenase from heart-muscle preparation which is also very unstable[11,14].

The final fresh preparation catalyzed the oxidation of 5.2, 2.9 and 0.026 μmoles DPNH/mg protein/min when ferricyanide, DCIP, and MB respectively were used as electron acceptor[*]. The activity with cytochrome *c* or lipoic acid was less than 1 %

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[*] The term DPNH oxidase used here denotes the oxidation of DPNH by molecular oxygen via the components of the respiratory chain. It was determined spectrophotometrically in the presence of $3\cdot10^{-5}$ M cytochrome *c*[9]. The DPNH dedrogenase activity with different electron acceptors in the soluble enzyme system was determined essentially by the following methods: DCIP[10]; ferricyanide[11] ($1.5\cdot10^{-4}$ M DPNH); MB[12] ($1.5\cdot10^{-5}$ M MB); cytochrome *c*[9]; and lipoic acid[4]. All tests were performed at pH 7.4, except in the system with lipoic acid which was at pH 5.9. The activity in the system with the heart-muscle preparation was determined in the presence of 10^{-3} M KCN. The initial rates at the specified concentration of electron acceptors are reported in the text.

of that with ferricyanide in contrast to the Keilin-Hartree preparation which exhibited strong activity as lipoic dehydrogenase. Mention should be also made that the Keilin-Hartree preparation did not show any activity of α-ketoglutaric dehydrogenase tested manometrically in the ferricyanide system according to the method of SANADI *et al.*[15] even in the presence of thiamine diphosphate, lipoic acid, coenzyme A, glutathione, DPN and $MgCl_2$. The high selectivity of the present enzyme toward electron acceptor recalls another property of soluble succinic dehydrogenase which reacts significantly only with N-alkylphenazines and ferricyanide[11, 14].

Preliminary experiments on reconstitution of an alkali-treated heart-muscle preparation[16] with the dehydrogenase described, or with diaphorase[17], or with Mahler's enzyme[17] failed to show restoration of the DPNH oxidase activity to a significant degree even in the presence of α-tocopherol, ubiquinone (coenzyme Q)* and/or sarcosomal lipids.

This enzyme is evidently different from diaphorase and DPNH-cytochrome *c* reductase in view of its different specificities for electron acceptors and fluorescence. Its role in the respiratory chain and its relationship with the other two enzymes as well as with the lipoflavoprotein reported by ZIEGLER *et al.*[18] and the DPNH oxidase of HUENNEKENS *et al.*[12] remain to be studied.

This work was supported by grants from the National Science Foundation and the U.S. Public Health Service. Published with approval of the Monograph Publication Committee, Research Paper No. 366, School of Science, Oregon State College.

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Received November 30th, 1959

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* Kindly supplied by Drs. E. R. Redfearn and R. A. Morton, University of Liverpool.

The adaptive formation of ubiquinone 30 (coenzyme Q_6) in yeast

Ubiquinone[1] or coenzyme Q*ref.*[2] has been found to be associated with electron transport in mitochondrial particles. Recently Lester and Crane[3] have found that organisms with a high aerobic metabolism contain much greater amounts of the quinone than anaerobes. Among different groups of organisms, the length of the isoprenoid side chain was found to vary widely. During studies on the biosynthesis of the quinone (ubiquinone 30 or coenzyme Q_6) in yeast, we noted that yeast grown under anaerobic conditions contained less quinone than yeast grown aerobically. This was also observed and reported by Lester and Crane[3]. We have found that the synthesis of the extra amounts of quinone appears to be an adaptation to oxygenation. This finding is in agreement with the important role postulated for the quinone in respiration and is analogous to the well known adaptive formation in yeast of respiratory enzymes and heme pigments to aerobiosis as observed by Slonimski[5]. Typical results on adaptive quinone formation over a time period are shown in Table I. It is seen that yeast grown under anaerobic conditions according to Klein[6] contains relatively small amounts of quinone. An increase of more than 5-fold in the level of quinone occurs when the yeast is suspended in a medium containing phosphate and

TABLE I

THE SYNTHESIS OF UBIQUINONE 30 (COENZYME Q_6) UNDER VARIOUS CONDITIONS

2.5 g of pressed Red Star yeast was grown anaerobically in 2.5 l 2 % peptone, 2 % dextrose and 1 % yeast extract for 16 h. The yield of wet weight of yeast varied from 28 to 30 g. After washing with distilled water, 20–25 g of yeast were added to 1 l 0.1 *M* KH_2PO_4 brought to pH 5.7 with NaOH and aerated by bubbling air through a coarse sintered-glass filter stick for various times at 30°. Additions to the aeration medium are as shown in the Table. Ubiquinone 30 was analyzed by saponification of the yeast in the presence of pyrogallol under N_2, followed by extraction 4 times with 0.5 vol. ether. Aliquots of the ether extract were evaporated and dissolved in ethanol and analyzed for ubiquinone 30 by measurement of the change in absorption at 275 mμ induced by the addition of KBH_4. This method gave values which were 5 % greater than those obtained by isolating the quinone on alumina column chromatography. Subsequent purification by paper chromatography showed that only ubiquinone 30 (coenzyme Q_6) was present. Values below 40 mg/kg dry weight are subject to an error of ± 15 %.

Expt. No.	*Addition to aeration medium*	*Time of aeration (h)*	*Dry wt. of yeast (g)*	*Ubiquinone 30 (coenzyme Q_6) mg/kg dry wt.*
1	none	0	4.04	53
	1.2 % glucose	3	5.90	105
	1.2 % glucose	5	5.50	163
	1.2 % glucose	7	5.58	163
2	none	0	4.72	17
	none	7	4.43	38
	0.1 % glucose	7	4.72	102
	0.5 % glucose	7	5.69	197
	0.2 % shikimic acid	7	4.42	91
	0.1 % glucose + 0.2 % shikimic acid	7	4.78	86
	0.2 % DL phenylalanine	7	4.51	69
	0.1 % glucose + 0.2 % DL phenylalanine	7	4.79	90
3	none	7	4.61	19
	0.1 % glucose	7	4.67	47
	0.5 % glucose	7	5.21	102
	0.2 % meso-inositol	7	4.49	64
	0.1 % glucose + 0.2 % meso-inositol	7	4.81	64
	0.2 % sodium acetate	7	4.72	55
	0.1 % glucose + 0.2 % sodium acetate	7	5.00	60

glucose and aerated for varying periods of time. In some experiments the increase was as much as 10-fold. Expt. 2 shows that the presence of glucose is essential. Addition of probable precursors of the benzoquinone moiety of ubiquinone in the presence of minimal amounts of glucose showed no significant effect in elevating the rate of synthesis. Compared to extra glucose, these substances, *e.g.* shikimic acid, phenylalanine, inositol were relatively ineffective. However, it is recognized that permeability factors may play some role here, and these findings must be considered tentative only. Of further interest is the finding that addition of 2,3-dimethoxy-5-methyl-1,4-benzoquinone, which constitutes the benzoquinone nucleus, in a concentration of 50 mg/l had no effect on the synthesis of ubiquinone. Rather it was usually inhibitory.

Recent work by HEBB *et al.*[7] has shown that the respiratory deficiency in the oxidation of succinate observed in yeast grown under anaerobic conditions was not due to decreased succinic dehydrogenase activity but rather to some defect in the overall succinate-cytochrome *c* reductase system. It seemed of interest to determine whether the defect in succinate-cytochrome *c* reductase activity in anaerobic yeast particles might be due to a lack of coenzyme Q. Particles from anaerobic yeast were prepared and assayed exactly as described by HEBB *et al.*[7] with very low succinic-cytochrome *c* reductase activity compared to those from aerobic yeast. Coenzyme Q_6 (ubiquinone 30) was isolated from yeast nonsaponifiable fractions by chromatography on alumina. The purity was checked spectrophotometrically and also by the chromatographic procedure of LESTER AND RAMASARMA[8]. The addition of quinone to the anaerobic particles showed no increase in succinic-cytochrome *c* reductase activity over control values. Addition of a lipid extract obtained by isooctane extraction of aerobic particles according to CRANE *et al.*[9] together with coenzyme Q_6 had no effect on the reduction of cytochrome *c*. Similarly no stimulatory effect was obtained when aurantiogliocladin* (2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone) was added. Thus it seems probable that lack of other factors in addition to the possible requirement of quinones may be responsible for the observed deficiency.

In view of the foregoing results on adaptive formation, it would appear that yeast might offer a desirable system for studying certain aspects of the biosynthesis of ubiquinone 30 (coenzyme Q_6). Further studies on yeast are in progress.

This investigation was supported by a Senior Research Fellowship (SF-199) of the U.S. Public Health Service to one of us (H.R.).

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* We are indebted to Dr. Karl Folkers of the Merck Sharp and Dohme Co. for this sample.

Received December 15th, 1959

Oxidative phosphorylation in sarcosomes from thoracic muscles of the American cockroach

It has been observed that mitochondria (sarcosomes) prepared from insect sources differ in several significant respects from mammalian heart or liver mitochondria. First, a requirement for serum albumin in the phosphorylation medium has been demonstrated repeatedly[1–5]. In some cases[1,4] the requirement appears to be absolute. Second, insect mitochondria oxidize the substrate α-glycerol phosphate at very rapid rates as compared with the rate of oxidation of citric acid cycle intermediates[6–9]. Third, phosphorylating respiration, which has been referred to by REMMERT AND LEHNINGER[10] as "loosely coupled", has been reported for the housefly[9]. Finally, the P/O ratios obtained with insect mitochondria[2,4,5,9] are quite low.

In the present communication, evidence is presented which bears on the last three of these points. The data were obtained using thoracic-muscle mitochondria from adult male American cockroaches, *Periplaneta americana* (L.). The mitochondria were prepared in 0.25 *M* sucrose following, in general, the technique described by SACKTOR[1] as modified by SACKTOR AND COCHRAN[9]. It was found (Table I) that roach sarcosomes are capable of oxidizing several metabolic substrates at significant rates. It is interesting to note that with this insect the Qo_2 for α-glycerol phosphate is approximately four times that of either Krebs-cycle intermediate tested or glutamate. This is a somewhat greater ratio than was reported for the housefly[9] when similar techniques were employed.

TABLE I

OXIDATIVE PHOSPHORYLATION AND RATES OF RESPIRATION IN MITOCHONDRIA FROM *P. americana* (L).

Incubation medium: $MgCl_2$, 20 μmoles; phosphate buffer, pH 7.4, 40 μmoles; glucose, 50 μmoles; hexokinase, 150 K.M. units; substrate, 60 μmoles; ADP, 10 μmoles; bovine serum albumin, 2%; KOH, 5 *M* in center well; mitochondria (2–3 mg protein); sucrose, 0.25 *M* to final vol. of 2.5 ml; temp., 25°; gas phase, air. Oxygen uptake was measured in a conventional Warburg apparatus; phosphorylation was determined by measuring inorganic phosphate present at the beginning and end of the experiment using the method of FISKE AND SUBBAROW[12].

Substrate	*ΔO μatoms*	*ΔP μmoles*	*P/O*	Qo_2 *μl O_2/mg protein/h*
α-Ketoglutarate	4.8 ± 0.4	13.6 ± 1.6	2.8	65.0
Succinate	5.0 ± 0.3	10.2 ± 1.4	2.0	67.2
Glutamate	5.0 ± 0.8	13.9 ± 2.6	2.8	67.2
α-Glycerol phosphate	5.2 ± 1.3	8.8 ± 2.6	1.7	253.1
None	0.8 ± 0.2	3.4 ± 1.5	—	10.0

Coupled with this substrate oxidation is significant esterification of inorganic phosphate (Table I). It can be seen that the P/O ratios approach those reported for mammalian mitochondria, with the exception of that for α-ketoglutaric acid. Even here, however, an occasional value of well over three has been obtained.

In other experiments it has been shown that the phenomenon of "loosely-coupled" respiration also occurs in the American cockroach. The rate of respiration of α-glycerol phosphate does not change significantly when the level of ADP is varied from zero through 12.5 μmoles per Warburg flask. Contrary to the results of SACKTOR AND

Abbreviation: ADP, adenosine diphosphate.

COCHRAN[9], however, it has been found that the rate of phosphate esterification also does not change appreciably over the entire range of ADP levels tested provided at least 0.5 µmole of exogenous ADP is present. As a result of this, very consistent P/O ratios (mean, 1.6) were obtained at all ADP levels tested. No phosphorylation occurred in the absence of ADP.

These experiments tend to support the concept that some, but not all, of the reported differences between insect and mammalian mitochondrial activity are real. It has been shown that high P/O ratios can be obtained with insect mitochondria. On the other hand, the rapid rate of oxidation of α-glycerol phosphate and the "loosely-coupled" phosphorylating respiration were confirmed. In this connection, it should be mentioned that SACKTOR has recently shown a 3- to 5-fold increase in the oxidation of α-glycerol phosphate when ADP was added to housefly-muscle minces[11]. This would seem to imply that respiratory control exists in the intact muscle, but not in isolated mitochondria.

We wish to thank Dr. R. W. ENGEL, Head of the Department of Biochemistry and Nutrition of the Virginia Polytechnic Institute for his interest and encouragement. The U.S. Public Health Service supported this work with a grant (No. E-2067) from the National Institute of Allergy and Infectious Diseases.

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Received October 23rd, 1959

Biochim. Biophys. Acta, 37 (1960) 562–563

On the mechanism by which progesterone stimulates galactose metabolism

Previous reports[1,2] have described the stimulation by progesterone of the oxidation of D-galactose in liver and small-intestine slices, and in the soluble fraction of liver homogenate. Insofar as the effect is manifest when UDPGal but not when UDPG is the substrate, it was concluded that a site of action of progesterone is at the level of the conversion of UDPGal into UDPG[2]. However since purified UDPGal 4-epimerase is not stimulated the effect is indirect. The mechanism of this hormone stimulation has now been partially delineated.

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It is known[3] that liver UDPGal 4-epimerase requires DPN and is inhibited by DPNH. Therefore it seemed reasonable that progesterone might elicit elevated epimerase activity by lowering the level of DPNH. The effect of progesterone in the presence of an oxidizable substrate such as propylene glycol on the reduction of DPN by an $(NH_4)_2SO_4$ fraction (40–60 % satn.) obtained from the soluble portion of rabbit-liver homogenate is shown in Fig. 1. It can be seen that progesterone is eight times more effective in decreasing the level of DPNH than would be predicted if it were acting stoichiometrically as an acceptor of electrons from DPNH. The hormone had no effect on the oxidation of DPNH by this preparation, nor did it effect the reduction of TPN.

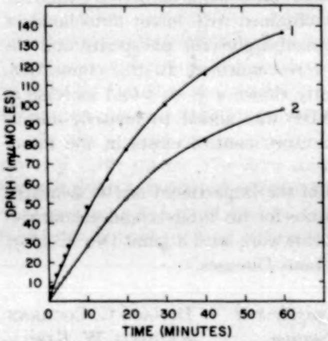

Fig. 1. Effect of progesterone on DPN reduction by the 40–60 % satd. $(NH_4)_2SO_4$ precipitate from the soluble fraction of rabbit liver. Reaction mixtures contained 300 mμmoles DPN and 0.4 mg protein [$(NH_4)_2SO_4$ precipitate] in a final volume of 0.5 ml 0.1 *M* glycine buffer, pH 8.8. In addition, reaction mixture (1) contained 5 μl propylene glycol and reaction mixture (2) contained 5 mμmoles progesterone dissolved in 5 μl propylene glycol. DPNH was measured by increase in absorbancy at 340 mμ.

Progesterone has little or no effect on twice-recrystallized horse-liver alcohol dehydrogenase*, calf-liver aldehyde dehydrogenase**, or aldehyde dehydrogenase activity obtained from an $(NH_4)_2SO_4$ fraction (0–40 % satn.) of the soluble portion of rabbit liver. However, it does exert a marked inhibition, at 10^{-5} *M*, on aldehyde dehydrogenase activity of the fraction of rabbit liver obtained between 40 and 60 % satn. with $(NH_4)_2SO_4$. The observed inhibitions were 25 %, 53 %, 66 % and 72 % with butylaldehyde, propionaldehyde, DL-glyceraldehyde and glycolaldehyde, respectively. In the latter case progesterone was 60 times more effective in decreasing the level of DPNH than would be predicted if it were acting stoichiometrically. It also inhibits rabbit-kidney aldehyde dehydrogenase. These observations suggested that it might be possible for the soluble fraction of kidney homogenate, a system in which galactose metabolism is normally not affected by progesterone, to become progesterone-responsive, if it were supplied with an aldehyde-generating system. Table I shows that galactose oxidation by heated kidney supernatant is not stimulated by the hormone, and that alcohol dehydrogenase in the presence of propylene glycol inhibits this oxidation. Progesterone largely overcomes this inhibition. Similar results were obtained when glycolaldehyde was substituted for propylene glycol in the absence of

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Abbreviations: UDPGal, uridine diphosphogalactose; UDPG, uridine diphosphoglucose; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH oxidized and reduced triphosphopyridine nucleotide.

* Obtained from the Worthington Biochemical Corporation.

** Prepared according to E. RACKER, *J. Biol. Chem.*, 177 (1949) 883.

TABLE I

PROGESTERONE EFFECT ON THE OXIDATION OF [1-^{14}C]D-GALACTOSE BY THE HEATED SOLUBLE FRACTION OF RABBIT-KIDNEY HOMOGENATES

The preparation of the kidney homogenate and the conditions of incubation were as described previously[2] except that kidney was substituted for liver and TPN was the only pyridine nucleotide added. 0.03 ml propylene glycol and 0.3 μmole progesterone (in propylene glycol) were added as indicated. Horse-liver alcohol dehydrogenase was obtained from the Worthington Biochemical Corporation.

Additions	*Total counts/min in CO_2 (counted at 50% efficiency)*	*Relative radioactivity in CO_2*
Propylene glycol (control)	2120	1.00
Progesterone in propylene glycol	2150	1.01
Alcohol dehydrogenase (excess)	2200	1.02
Propylene glycol and alcohol dehydrogenase	1210	0.57
Progesterone in propylene glycol and alcohol dehydrogenase	1865	0.88

alcohol dehydrogenase. This action of the hormone is ascribed to inhibition of the oxidation of aldehyde. It appears, then, that the reason that galactose metabolism in kidney is normally not influenced by progesterone *in vitro* may be related to the low level of alcohol dehydrogenase activity[4] present in this tissue.

Our observations are not in accord with the interpretations of ISSELBACHER AND McCARTHY[5] who suggest that progesterone stimulates galactose oxidation at the level of the phosphogluconic acid oxidative pathway by stoichiometrically regenerating TPN from TPNH[6,7] in the course of reduction of the 4,5-double bond by the steroid reductases. This formulation is not consistent with the effect of progesterone on galactose oxidation by the heated soluble fraction of kidney (Table I), a tissue which lacks steroid reductases[6]. Recent[2] and present results indicate that progesterone, by inhibiting DPNH formation, can stimulate galactose oxidation at the level of the UDPGal 4-epimerase reaction. Whether this is the sole mechanism by which the hormone stimulates galactose oxidation is not yet known.

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Received November 30th, 1959

Steroid C-17 hydroxylation in guinea-pig-adrenal homogenates

Steroid C-11 and C-21 hydroxylations in adrenocortical tissue are oxidative reactions that require TPNH and atmospheric oxygen[1-4]. The requirements for adrenocortical C-17 hydroxylation remain to be established. Investigations of this reaction in some species have been hampered by the loss of C-17 hydroxylating activity that attends the preparation of cell-free systems[4]. Utilizing the adrenal glands of a readily-available animal, the guinea pig, we have prepared cell-free systems in which the reliable occurrence of C-17 hydroxylation permits the study of this reaction.

Minced adrenal glands were homogenized in a loosely-fitting (*i.e.* 0.5 mm clearance between pestle and tube all round) apparatus. The medium for homogenization, the differential centrifugation and the methods employed for the extraction of the conversion products formed during incubation of the 15,000 × *g* supernatant were identical to those of RYAN AND ENGEL[4]. The conversion products were measured as Porter-Silber chromogens or separated by paper chromatography in the systems toluene–propylene glycol and formamide–propylene glycol.

A 15,000 × *g* supernatant prepared from guinea-pig-adrenal homogenates contains both the C-17 and the C-21 hydroxylation systems and we have not been able as yet to isolate the C-17 system. Consequently, the model reaction followed in our studies was the conversion of progesterone to 17,21-dihydroxyprogesterone (Reichstein's Substance S). This conversion product was located on paper chromatograms by ultraviolet absorption and reaction with blue tetrazolium. Its mobility alone, when admixed with Substance S and following acetylation, was that to be expected of Substance S. Using the KBr-pellet technique, the acetylated material was subjected to infrared spectroscopic analysis and the resulting spectrum compared to one similarly obtained from 600 μg of authentic Substance S acetate. Although the spectrum obtained from the conversion product was a weak one, it did contain 23 of the 25 absorption maxima found in range 3500 to 775 cm^{-1} in the spectrum of the authentic compound. The two missing bands, at 1200 and 930 cm^{-1}, were present only weakly in the reference spectrum. By the above paper chromatographic criteria, 11-desoxycorticosterone and Δ^4-androstene-3,17-dione were tentatively identified as other conversion products of progesterone. Evidence for the presence of 17α-hydroxyprogesterone among the conversion products was usually lacking, and no C-11 hydroxylation occurred so long as a loosely-fitting homogenizer was used.

The optimal conditions for the conversion of progesterone to Substance S were found to be a substrate concentration of 0.001 *M* and an incubation time of 1.5 to 2 h. Under these conditions, 7.2–9.3% of the progesterone was converted to Reichstein's Substance S. The conversion did not proceed to any detectable extent in an atmosphere devoid of oxygen. Some C-21, but no C-17, hydroxylation of progesterone occurred in the absence of exogenously-supplied cofactors. C-17 hydroxylation occurred when DPN (0.005 *M*) and ATP (0.002 *M*) were added; these cofactors could be wholly replaced by TPNH (0.005 *M*). The guinea-pig adrenal gland stores no detectable quantities of Substance S nor is any formed *in vitro* unless an appropriate steroid substrate is supplied. As a precursor of Substance S in our system, progesterone can be replaced by Δ^5-pregnen-3β-ol-20-one but not by 11-desoxycorticosterone. The

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Abbreviations: TPNH, reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate.

Δ^{16} and 16,17-epoxido congeners of Δ^5-pregnen-3β-ol-20-one were not converted to Substance S.

Centrifugation of the 15,000 × *g* supernatant at 105,000 × *g* produced a sediment that would not convert progesterone to Substance S upon the addition of ATP and DPN or TPN in an atmosphere of room air. The conversion proceeded only upon the addition of TPNH.

It would thus appear that the enzymic systems in the adrenal cortex responsible for the hydroxylation of carbons 11,17 and 21 of progesterone have identical requirements: TPNH and atmospheric oxygen.

This work was supported in part by a grant (C-2121) from the National Institutes of Health, U.S. Public Health Service. Thanks are due to Dr. Samuel Solomon for carrying out the infrared spectroscopic analyses and for much helpful advice in the conduct of this work.

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Received November 20th, 1959

Hydrolysis of synthetic peptides by collagenase

Several reports have appeared recently concerning the substrate specificity of collagenase[1–4]. It is now generally agreed that the specificity requirement may be expressed as Pro-R-R′-Pro.

In order to specify the nature of the substrate specificity in more detail, a number of peptides were synthesized and their hydrolysis by collagenase was investigated. The solution of a substrate peptide of about 1 mg/ml was incubated at 37° with collagenase at 0.05 mg/ml for 5 h. Dinitrophenylation followed by paper chromatography was employed for the detection of hydrolysis. The collagenase used was isolated from a 40-h culture filtrate of *Clostridium histolyticum*, and purified by fractional precipitation with ammonium sulfate and by starch-column electrophoresis at a pH of 8.5, as described previously[2].

From the results in Table I, the following conclusions may be drawn:

(1) The imino group of the first proline residue in the scheme of Pro-R-R′-Pro must be masked.

(2) The masking of the carboxyl group of the last proline residue is not always necessary.

(3) It is established that both proline residues in the scheme may be replaced by a hydroxyproline residue.

The masking with DNP of the imino end of the first proline residue is sufficient for the digestion by collagenase. This indicates that no carbonyl group is required

TABLE I

HYDROLYSIS OF SYNTHETIC PEPTIDES BY COLLAGENASE

Peptides readily hydrolyzed		*Peptides slowly hydrolyzed*		*Peptides not hydrolyzed*
Peptides	*Decomposition products*	*Peptides*	*Decomposition products*	*Peptides*
Cbz·Pro·Leu·Gly·Pro	Cbz·Pro·Leu Gly·Pro	Gly·Pro·Leu·Gly·Gly·Pro	Gly·Gly·Pro Gly·Pro·Leu	Gly·Pro·Gly·Pro Pro·Leu·Gly·Pro
DNP·Pro·Leu·Gly·Pro	DNP·Pro·Leu Gly·Pro	Cbz·Ala·Gly·Pro–NH_2	Cbz·Ala Gly·Pro–NH_2	Gly·Gly·Leu·Gly·Pro
Gly·Pro·Leu·Gly·Pro–NH_2	Gly·Pro·Leu Gly·Pro–NH_2	Cbz·Leu·Gly·Pro–NH_2	Cbz·Leu Gly·Pro–NH_2	Gly·Pro·Leu·Gly·Gly Gly·Pro·Gly·Leu·Pro
Gly·Pro·Leu·Gyl·Pro	Gly·Pro·Leu Gly·Pro			Gly·Pro·Leu·Leu·Pro
Gly·Pro·Leu·Gly·Hypro	Gly·Pro·Leu Gly·Hypro			Cbz·Pro·Gly·Gly·Pro
Cbz·Gly·Pro·Leu·Gly·Pro	Cbz·Gly·Pro·Leu Gly·Pro			

next to the imino nitrogen of the proline residue. It seems that the only requirement is that the nitrogen atom has no hydrogen directly bound to it.

As regards the rule (2) the fact found by GRASSMANN *et al.*[4] that Cbz·Pro·Gly·Gly·Pro was not hydrolyzed by collagenase unless the carboxyl end of the last proline was blocked is noteworthy. Because of the difficulty in crystallizing the kind of peptides studied here we had to make all the peptides first with free carboxyl ends. We are in the process of making their amides and studying collagenase digestion.

HEYNS *et al.*[3] suggested the possibility that the proline residue required by the collagenase specificity may be replaced by a hydroxyproline residue, and GRASSMANN *et al.*[4] experimentally established that the first proline in the above-mentioned scheme may be replaced by a hydroxyproline. It was established in the present study that the last proline in the scheme may also be replaced by a hydroxyproline.

For R′ residue in the scheme, glycine is allowed but leucine is not. By hydrolyzing pure tendon collagen with collagenase, MANDL[5] obtained about 10 % of alanine in the N-termini of the decomposition product. Therefore, it is suggested that R′ may possibly be an alanine residue.

Taking all the results heretofore into consideration, the scheme for the substrate specificity of collagenase may better be represented as P-R-R′-P. P stands for a proline or a hydroxyproline residue.

There were, however, some results in which the rule of P-R-R′-P was not followed. HEYNS *et al.*[3] reported that Cbz·Ala·Gly·Pro–NH_2 was hydrolyzed by collagenase. This result was verified in the present study. The fact that Gly·Pro·Leu·Gly·Gly·Pro was hydrolyzed by collagenase is interesting in connection with the fact found by GRASSMANN *et al.*[4] and verified here that Cbz·Pro·Gly·Gly·Pro was not hydrolyzed. The hydrolysis of those peptides which did not belong to the P-R-R′-P type was generally slower than the hydrolysis of those peptides which did belong to the type.

In the course of the present study, it was found that Cbz·Gly·Pro·Leu·Gly·Pro was the best standard substrate for collagenase among the peptides used heretofore. Free peptides of P-R-R′-P type and their amides particularly were quite hygroscopic and deliquescent. Even their carbobenzoxy derivatives were hard to crystallize because of the effect of humidity. The above-mentioned substrate is readily crystallizable from ethyl acetate and is stable enough to stand in the open air. The determination of its decomposition products by collagenase is simple by titration or colorimetry.

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Received November 13th 1959

Abbreviations: Cbz, carbobenzoxy; DNP, dinitrophenyl-.

Inhibition de la lipase pancréatique par le diéthyl-*p*-nitrophényl phosphate en emulsion

Un récent travail[1] a montré que la lipase pancréatique de porc est une estérase particulière, incapable d'agir sur les esters en dispersion moléculaire, mais capable d'agir sur ces mêmes esters dès qu'ils se présentent à l'état émulsifié. La lipase exerce donc son action au niveau d'une interface sur laquelle elle s'adsorbe d'ailleurs réversiblement[2]. L'estérase hépatique, considérée comme une estérase typique, hydrolyse par contre les dispersions moléculaires d'esters. La création d'une interface ne modifie pas son activité[1].

Cette propriété fondamentale de la lipase vis à vis de ses substrats se manifeste-t-elle aussi avec ses inhibiteurs[2,3]? Le but de la présente note est d'aborder l'étude du problème avec l'un des membres (le diéthyl-*p*-nitrophénylphosphate ou E 600) du groupe des organophosphates dont l'effet inhibiteur par réaction stoechiométrique avec les estérases est bien connu.

La lipase purifiée (C_3[4,5]; 200–700 unités) est traitée à 37° et pH 7.6 (tampon phosphate ou tris(hydroxyméthyl)aminométhane 0.1 *M*), soit par une solution aqueuse de E 600, soit par une émulsion de cette substance dans la gomme arabique à 2 %. Des témoins sont préparés en remplaçant la solution ou l'émulsion de E 600 par un égal volume d'eau ou de gomme arabique à 2 %. On prélève de temps en temps des échantillons où l'on dose[5] l'activité lipasique restante. On calcule enfin l'effet inhibiteur en tenant compte, grâce aux témoins, de l'inactivation spontanée de la lipase. Cette inactivation est très lente dans les conditions de nos expériences.

La Fig. 1 indique les résultats obtenus avec une solution de E 600 dont la concentration finale est 30.7 % de la saturation à 37° (0.307 S) et avec deux émulsions de cette substance (concentrations finales: 1.07 et 7.78 S).

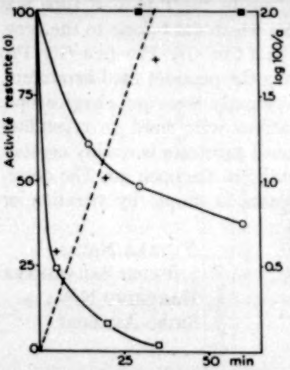

Fig. 1. Action du diéthyl-*p*-nitrophénylphosphate sur la lipase pancréatique. ■ : Solution 0.307 S; ○ et □ : Emulsions 1.07 et 7.78 S, respectivement. Les courbes en traits pleins illustrent la cinétique de l'inhibition et doivent être lues avec les ordonnées de gauche. La droite en pointillé correspond à la fonction $\log 100/(100-x) = Kt$ que doit suivre la réaction d'inhibition si elle est d'ordre 1. Elle est lue avec les ordonnées de droite.

Les courbes de la Fig. 1 montrent:

1. Contrairement à ce que signale ALDRIDGE[6], E 600 en dispersion moléculaire n'inhibe pas la lipase, tout au moins dans les conditions de nos essais. Cette contradiction est d'ailleurs plus apparente que réelle car l'inhibition constatée par Aldridge est très lente. Elle est en outre réversible, ce qui suggère que son mécanisme est

différent de celui de la réaction covalente caractéristique des organophosphates.

2. Dès qu'une partie de l'organophosphate est en émulsion, un effet inhibiteur considérable se manifeste. Relativement lent et incomplet quand la saturation vient d'être dépassée (1.07 S), cet effet devient rapide et total quand la quantité de E 600 émulsifié est importante (7.78 S). Dans ce dernier cas, la réaction d'inhibition est approximativement d'ordre 1 (droite en pointillé de la Fig. 1).

On pourrait objecter ici que la lipase n'est plus active au cours de certains de nos essais, pour la seule raison qu'elle est adsorbée. Si, après avoir réalisé l'expérience précédente où la lipase est presqu'entièrement inhibée (émulsion 7.78 S), on dilue avec du tampon jusqu'à dissolution complète de E 600, on ne constate en réalité aucun renouveau d'activité. La lipase est donc inhibée de façon permanente, même après remise en solution.

Ces résultats, obtenus avec un seul inhibiteur, ne permettent pas de tirer des conclusions définitives. Ils suggèrent néanmoins dès maintenant que la lipase en solution est une protéine inactive et qu'elle devient un enzyme au moment de l'adsorption à une interface. On admet à l'heure actuelle que le centre actif des zymogènes pancréatiques est formé grâce à certaines modifications de la structure tertiaire dues à la rupture de liaisons peptidiques. Une hypothèse de travail assez séduisante serait de supposer que le centre actif de la lipase est formé grâce à une "dénaturation" partielle due à l'adsorption. Notons pour terminer que l'inhibition spécifique des lipases par des inhibiteurs en émulsion devrait permettre de mieux différencier les activités lipasique et "estérasiques" des tissus[1,6,7].

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Received December 8th, 1959

Biochim. Biophys. Acta, 37 (1960) 570–571

Direct chromatography of serum lipids without solvent extraction

It is common practice to make organic-solvent extracts of lipids from tissues and body fluids before subjecting them to chromatographic analysis. However, it seemed plausible that certain lipid-containing body fluids such as plasma, serum, and cytoplasm and finely dispersed particles such as mitochondria and microsomes, although existing in a predominantly aqueous medium, might be chromatographed directly. The studies on serum which are reported in this paper demonstrate that this is indeed the case. 10–20 μl of serum are applied to the paper, allowed to dry and then chromatographed in the usual manner[1,2]. The solvents are sufficiently polar to rupture the lipid-protein bonds and cause the lipids to migrate as discrete spots. The proteins

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remain at the point of application (origin). The chromatograms can be washed in water without appreciable loss of lipid but with a loss of most of the non-lipid water-soluble compounds (salts, amino acids, nucleotides, phosphate esters and sugars).

This study was done on human serum obtained by clotting and centrifuging whole blood. Chromatography was carried out on paper (20 × 21 cm sheets) impregnated with silicic acid as described previously[3]. 10, 15 and 20 μl of serum are placed at the starting line. 8–12 samples can be run on each paper. The sheets are rolled into a cylinder and held together by nichrome wire. Chromatography is carried out in large-mouth, screw-cap, 2-quart Mason jars. Solvent A (50 ml) for separating the phosphatides is diisobutyl ketone–acetic acid–water (40:20:3). Solvent B (50 ml) for separating the non-phosphatides (cholesterol, cholesterol esters, glycerides and fatty acids) is *n*-heptane–diisobutyl ketone (96:6)[4]. Ascending chromatography is carried out at 23° in a constant-temperature room. Solvent A requires 4–5 h whereas solvent B requires 1–$1^{1}/_{4}$ h (allowed to ascend 16–18 cm). The chromatograms are air-dried for 30 min and then stained with Rhodamine 6G[2]. Chromatograms run in solvent B are sprayed lightly with solvent A and dried for 30 min before staining. This technique was found to enhance the detection of the non-phosphatides. Typical chromatograms are shown in Figs. 1 and 2.

The present method allows for the rapid qualitative analysis of nearly all the individual lipids of serum and requires only 20–40 μl of serum. Since 8–12 serum samples can be run on one sheet of paper and since many Mason jars can be employed easily and inexpensively, the method is well suited for screening a large number of blood samples and hence should have routine clinical application. In the analysis of 100 random serum samples (obtained from the Blood Laboratory), the most variable component of the fasting morning blood samples was the triglyceride fraction.

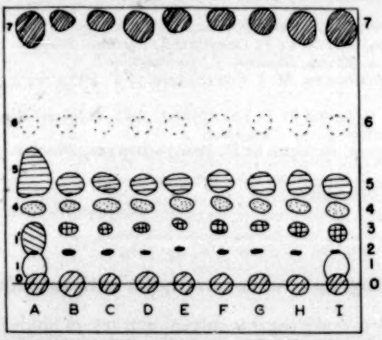

Fig. 1. Tracings of chromatograms of human serum phosphatides. 10 μl of fasting morning samples of serum were applied directly to the paper. Chromatography was carried out in solvent A as explained in the text. 0, serum proteins which are bound to the paper; 1, 1', and 2 are unidentified; 3, mixture of lysolecithin and inositol phosphatide (in some cases these are separated); 4, sphingomyelin (a trace of lysophosphatidyl-ethanolamine may occur in this spot); 5, lecithin; 6, phosphatidylethanolamine (this minor spot is not easily detected with Rhodamine 6G but is observed after reaction with ninhydrin); 7, the non-phosphatides. Spots nos. 1 and 1' are colored and may be bile pigments. The staining reaction with Rhodamine 6G is as follows: spot no. 0, yellow-purple; 1, 1', brown-purple; 2, purple; 3, lower part is purple, upper part is yellow; 4–7, yellow. Serum samples A and I were taken from patients having jaundice. The high content of lecithin in sample A is apparent.

Components believed to be diglycerides were also observed to be minor but normal lipid constituents of serum. The free fatty acids in serum occur in small amount and are not detectable.

The present method also has the following advantages: (a) since the same volume of serum is used the relative amount of each lipid in various samples can easily be determined and the amount of each lipid per unit volume of serum is directly obtainable; (b) time studies on the effect of many variables on all the serum lipids can easily be carried out; (c) the action *in vitro* of enzymes on serum lipids can be studied; (d) the method can be made quantitative by elution of the spots and analyzing for P, cholesterol, or ester; (e) the spots are small, discrete and well-suited for autoradiographic analysis; (f) the method is very effective for piloting column fractionations and testing purity of lipids.

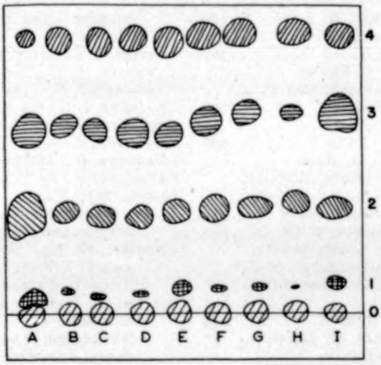

Fig. 2. Tracings of chromatograms of human serum non-phosphatides. 10 μl of the same sera given in Fig. 1 were used for these analyses. Chromatography was carried out in solvent B as explained in the text. 0, phosphatides + serum proteins; 1, diglycerides (tentative), 2, free cholesterol, 3, triglycerides; 4, cholesterol esters. Spot no. 0 stains yellow-purple and spots 1–4 stain yellow with Rhodamine 6G. The high content of free cholesterol, and triglyceride in sample A and triglyceride in sample I and the very low content of cholesterol esters in sample A is apparent.

This method is presently being used to study the effect of venom phospholipase A and pancreatin on serum and plasma lipoproteins and also the metabolic fate of specific lipids after their addition to mitochondrial, microsomal and cytoplasmic systems.

This work was supported in part by a grant (H 2063) from the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

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Received December 15th, 1959